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(54) Title: USE OF HEPARIN–BINDING ANTAGONISTS IN THE INHIBITION OF BRADYKININ RELEASE

(57) Abstract

The invention is related to a method for treating or preventing a disorder resulting from the release of bradykinin in said mammal, particularly a mammal which produces an HBP which binds to an HBP antagonist, e.g. a monoclonal antibody that binds to at least one epitope of human HBP, comprising administering to said mammal an HBP antagonist in an amount effective to decrease the release of bradykinin in a mammal. Furthermore, the invention is directed to methods and kits for determining if a mammal produces HBP that binds to an HBP antagonist, e.g., a monoclonal antibody that binds to at least one epitope of human HBP and a method for detecting an antagonist of HBP.

USE OF HEPARIN-BINDING ANTAGONISTS IN THE INHIBITION OF BRADYKININ RELEASE

FIELD OF THE INVENTION

5   The invention is related to a method for treating or preventing a disorder resulting from the release of bradykinin in a mammal, particularly a mammal which produces a heparin-binding protein which heparin-binding protein binds to a heparin-binding protein antagonist comprising administering to said mammal a heparin-binding protein antagonist in an amount effective to decrease the release of bradykinin in a mammal. Furthermore, the invention is di-
10   rected to methods and kits for determining if a mammal produces heparin-binding protein that binds to a heparin-binding protein antagonist and a method for detecting an antagonist of heparin-binding protein.

BACKGROUND OF THE INVENTION

15

Inflammation
An acute inflammatory response consists of several features, including changes in vascular caliber and tone, as well as an increase in vascular permeability that results in the formation of a protein-rich exudate (Lewis in Mediators of Inflammation, Wright, Bristol, U.K., 1986).
20   As soon as neutrophils (PMNs) receive chemotactic signals, they marginate and adhere to the vascular endothelial cells via specific adhesion molecules synthesized on both the endothelial and neutrophil surfaces. After neutrophils are attached onto endothelial cells, there is an opening of the endothelial gaps that induce vascular permeability and permit the migration of the neutrophils into the interstitial tissue space.
25

The contact-phase system encompasses three enzymatic factors, factor XI (F XI), factor XII (F XII) and plasma prokallekrein (pre-kallekrein) (PK) and the nonenzymatic cofactor, H-kininogen (HK) , which forms equimolar complexes with F XI and PK, respectively. The contact phase is present on monocytes, fibroblasts and neutrophils. Specific binding sites ex-
30   posed by endothelial cell and noncellular negatively charged surfaces such as kaolin, collectively referred to as the "contact phase", allow the local assembly of the critical components. The conversion of the zymogen, F XII to active enzyme, F XIIa, activates the contact-phase system itself (Colman et al., 1986, Crit. Rev. Oncol. Hematol. 5: 57-85). Reciprocal activation of surface-bound F XII and PK anchored to the surface via HK generates F XIIa and PKa

thereby amplifying the initial signal. Factor XIIa can then activate Factor XI and the intrinsic pathway of coagulation is initiated. It is known that PK also hydrolyzes HK to produce the potent nonapeptide, bradykinin (Kaplan and Silverberg, 1987, Blood 70:1-15). Kinins are considered to be the primary mediators of inflammatory process that produce pain, induce

5   vasodilation and increase vascular permeability due to a direct effect on endothelial cells, causing them to retract and permit both the migration of neutrophils and transudation of plasma constituents (Oyvin et al., 1970, Experentia 26:843-844). Local production of pros- taglandins and nitric oxide, NO may also be involved (Hall, 1992, Pharmacol. Ther. 56:131- 190). Therefore, activation of the contact-phase system may result in a number of deleteri-

10   ous effects, e.g., inflammation, septic shock, adult respiratory distress syndrome, dissemi- nated intravascular coagulation, postoperative bleeding from cardiovascular surgery.

Specifically, the contact phase system may be activated when neutrophils bind to endothelial cells, by the presence of endotoxins and by bacterial infection (reviewed in Colman et al.,

15   1997, Blood 90:3819-3843). For example, it has been found that in sepsis, activation of fac- tor XII and prekallikrein result in cleavages that activate them to enzymes that rapidly react with C1-inhibitor to form factor XIIa-C1-inhibitor and kallikrein-C1-inhibitor complexes. A sig- nificant increase in kallikrein-C1-inhibitor complex formation is observed in simulated cardio- pulmonary bypass.

20

It is found that aprotinin, an inhibitor of both plasmin and plasma kallikrein, reduces blood loss after cardiac operations and decreases the elevated postoperative bleeding time. Spe- cifically, aprotinin is found to decrease both kallikrein-C1-inhibitor and C1-C1-inhibitor com- plexes in a simulated extracorporeal bypass model (Wachtfogel et al., 1993, J. Thorac. Car-

25   diovasc Surg. 106:1). When aprotinin is added to cardiopulmonary bypass circuits that are perfused with whole blood anticoagulated with heparin, aprotinin actually complements the action of heparin (Bannan et al., 1998, Brit. J. Haem. 101:455-461). Therefore, aprotinin has an additional haemostatic beneficial effects to those found with heparin-bonded circuits.

30   Aprotinin also increases endothelial cell viability in hypoxic cold storage conditions when such cells are stored in organ preservation solutions and improves lung and myocardial preservation in whole organ models (Sunamori et al., 1991, Ann. Thor. Surg. 52:971-978 and Roberts et al., 1998, Ann. Thorac. Surg. 66:225-230). Furthermore, while bradykinin is found to increase vascular permeability, aprotinin decreased this vascular permeability and

neutrophil count (O'Brien et al., 1997, Can. J. Physiol. Pharmacol. 75:741-749 and Dwenger et al., 1995, Eur. J. Clin. Chem. Clin. Biochem. 34:207-214).

5 **Heparin-Binding Protein**

The covalent structure of two closely related proteins isolated from peripheral neutrophil leukocytes of human and porcine origin have recently been determined (cf. H. Flodgaard et al., 1991, Eur. J. Biochem. 197: 535-547; J. Pohl et al., 1990, FEBS Lett. 272: 200 ff.). Both proteins show a high similarity to neutrophil elastase, but owing to selective mutations of the ac-
10 tive serine 195 and histidine 57 (chymotrypsin numbering (B.S. Hartley, "Homologies in Serine Proteinases", Phil. Trans. Roy. Soc. Series 257, 1970, p. 77 ff.)) the proteins lack protease activity. The proteins have been named human heparin-binding protein (hHBP) and porcine heparin-binding protein (pHBP), respectively, owing to their high affinity for heparin.

15 Schafer et al., (Schafer et al., 1984, Infect. Immun. 53:651) have named the protein cationic antimicrobial protein (CAP37) due to its antimicrobial activity. HBP binds strongly to the lipid A component of LPS and endotoxin ($K_{ass} = 0.8 \times 10^9 \ M^{-1}$). It has been suggested that the bactericidal effect of HBP is due to binding of lipid A (Petersen et al., 1993, Eur. J. Biochem. B214: 271-279., Flodgaard et al., 1994, J. Cell. Biochem. Suppl. *18A*: Abstr. E505; Pereira
20 et al., 1993, Proc. Natl. Acad. Sci. USA 90: 4733-4737). The putative lipid A/LPS binding site of native HBP is located as an uncharged patch between a basic and an acidic patch on HBP, and comprises residues 20-26 and 41-43. In the lipid A/LPS binding site, Phe25, Cys26, Cys42, and Phe43 form a hydrophobic pocket suitable for binding of either the fatty acid chains or a glycosaminyl sugar ring of lipid A. Next to this pocket, an ionic and hydro-
25 philic pocket (Asn20, Gln21, and Arg23) is situated, which would be well suited for binding of a glycosaminyl linked phosphate group of lipid A (Iversen et al., 1997, Nature Struct. Biology 4: 265-268).

Furthermore, in animal models of fecal peritonitis, HBP treatment has been shown to rescue
30 mice from an otherwise lethal injury (Mercer-Jones et al., 1996, In Surgical Forum, pp. 105-108 and Wickel et al., 1997, In 4th International Congress on the Immune Consequences of Trauma, Shock and Sepsis, Munich, Germany, pp. 413-416). It has been proposed that heparin-binding protein or LPS-binding portions thereof may be used to treat septic shock (WO95/28949, U.S. Pat. Nos. 5,458,874, 5,607,916 and 5,650,392).

HBP was originally studied because of its antibiotic and LPS binding properties (Gabay et al., 1989, Proc. Natl. Acad. Sci. U.S.A. 86:5610-5614 and Pereira et al., 1993, Proc. Natl. Acad. Sci. USA 90: 4733-7). However, accumulating evidence now supports the concept that HBP, in addition to its bactericidal role, is involved in the progression of inflammation due to its effect on the recruitment and activation of monocytes (Pereira et al., 1990, J. Clin. Invest. 85:1468-1476 and Rasmussen et al., 1996, FEBS Lett. 390:109-112), recruitment of T cells (Chertov et al., 1996, J. Biol. Chem. 271: 2935-2940).

HBP has been found to induce contraction in endothelial cells and fibroblasts (Østergaard and Flodgaard, 1992, J. Leuk. Biol. 51: 316-323). In relation, WO 93/05396 discloses a method for screening for HBP inhibitors by incubating HBP or a cell producing HBP with a substance suspected of being an HBP inhibitor and with tissue or cells that are capable of interacting with HBP; decreased interaction (e.g. endothelial cell contraction, indicates that the substance is an HBP inhibitor.

Application WO99/26647 discloses the use of heparin-binding protein for the modulation or prophylaxis of apoptosis of mammalian cells. This application also discloses that HBP rescues rat insulinoma cells from IL-1 induced apoptosis.

Human heparin-binding protein but not porcine heparin-binding protein has also been found to bind to aprotinin (BPTI) (Petersen et al., Eur. J. Biochem. 214:271-279). Specifically, BPTI is still capable of binding to HBP with $K_d = 0.1 \times 10^{-6}$ M (Petersen et al., 1993, Eur. J. Biochem. B214: 271-279). The P1 specificity of HBP has been determined to be primarily Lys or Leu (Kiczak et al., 1999, Biol. Chem. 380: 101-105). The most prominent residues in HBP which influences the binding of BPTI have been suggested to be Gly169, Gly175, Ser192, and Asp201, corresponding to Asp189, Ser195, Gly216, and Asp226 in trypsin (Petersen et al., 1993, Eur. J. Biochem. B214: 271-279). Kiczak et al., 1999, Biol. Chem. 380:101-106 constructed a library of mutants in the P1 side chain of aprotinin using phage display methods. They found that HBP shows a strong affinity for P1 Lys as well as for the uncharged P1 amino acids, Leu, Thr, Met, Gln.

5

SUMMARY OF THE INVENTION

It has surprisingly been found that heparin-binding protein (HBP) serves as a signaling link in neutrophil-induced vascular leakage and activation of the contact phase system with con-
5   comitant formation of bradykinin and that it specifically plays a role in the PK mediated cleavage of HK to obtain the bradykinin sequence. Additionally, it has been found that antagonists of HBP decrease the permeability of endothelial cells. As defined herein an "HBP antagonist" is a substance that binds to heparin-binding protein and inhibits the action of heparin-binding protein.
10

The invention is directed to a method for treating or preventing disorders resulting from the release of bradykinin in a mammal, particularly a human patient, particularly a mammal that produces HBP that binds to an HBP antagonist, comprising administering to said mammal in need thereof, a mammalian heparin-binding protein antagonist in an amount effective to mo-
15   dulate or decrease release of bradykinin in said mammal. Such disorders include but are not limited to systemic inflammatory response syndrome, ischemia reperfusion, anaphylaxis and allograft rejection. Such disorders may also include adult respiratory distress syndrome a side effect of systemic inflammatory response syndrome. Anaphylaxis may occur from inappropriate activation of PMNs during cardiopulmonary bypass, lung surgery, head trauma
20   and major whole body trauma. The modulation or decrease in release of bradykinin is obtained by preventing contact of HBP with endothelial cells and/or with the contact-phase system. In a specific embodiment, the HBP antagonist modulates or decreases the kallikrein mediated cleavage of H-kininogen to obtain the bradykinin sequence. The invention is further directed to the use of an HBP antagonist for the preparation of a medicament useful for
25   the treatment of systemic inflammatory response syndrome, ischemia reperfusion, anaphylaxis and allograft rejection in a patient having a HBP that binds to a HBP antagonist. HBP antagonists may further be used to treat or for the preparation of a medicament useful for the treatment of a complication of systemic inflammatory response syndrome, adult respiratory distress syndrome.
30

In a specific embodiment, the invention is directed to a method for preventing disorders resulting from the release of bradykinin in a mammal, particularly a human patient, particularly a mammal that produces HBP that binds to an HBP antagonist, comprising administering to said mammal in need thereof a Kunitz-type serine protease inhibitor domain or analog or de-

rivative thereof that binds to HBP, in an amount effective to modulate or decrease release of
bradykinin in said mammal.

In another specific embodiment, the invention is directed to method for treating or preventing
5   disorders resulting from the release of bradykinin in a mammal, particularly a human patient,
particularly a mammal that produces HBP that binds to a monoclonal antibody that binds to
at least one epitope on HBP, wherein said epitope binds to prekallikrein-H-kininogen com-
plex and activates release of bradykinin, comprising administering to said mammal in need
thereof a monoclonal antibody that binds to at least one epitope on HBP, wherein said epi-
10   tope binds to prekallikrein-H-kininogen complex and activates release of bradykinin, in an
amount effective to modulate or decrease release of bradykinin in said mammal.

The invention is further directed to methods for detecting an antagonist of HBP. In one em-
bodiment, said method comprises: (a) culturing endothelial cells in the presence of HBP and
15   in the presence and absence of a substance suspected of being said antagonist and (b) de-
tecting any effect of said substance on permeability of endothelial cells, wherein decreased
permeability of said endothelial cells as compared to permeability of said cells when incu-
bated in the presence of HBP without said substance indicates that said substance is an an-
tagonist. In another embodiment, said method comprises (a) incubating HBP with a first
20   substance that interacts with HBP and a second substance suspected of being a HBP an-
tagonist and (b) detecting any effect of said second substance suspected of being an an-
tagonist on interaction of HBP with said first substance.

In a specific embodiment, the invention is directed to methods for identifying a monoclonal
25   antibody that binds to a least one epitope of HBP, particularly human HBP, wherein said epi-
tope binds to prekallikrein-H-kininogen complex and activates release of bradykinin. In one
embodiment, the method comprises: (a) culturing endothelial cells in the presence of HBP
and in the presence and absence of a monoclonal antibody suspected of binding to at least
one epitope of HBP, particularly human HBP, wherein said epitope binds to prekallikrein-H-
30   kininogen complex and activates release of bradykinin, and (b) detecting any effect of said
substance on permeability of endothelial cells, wherein decreased permeability of said endo-
thelial cells as compared to permeability of said cells when incubated in the presence of HBP
without said antibody indicates that said antibody is a monoclonal antibody that binds to at
least one epitope on HBP, wherein said epitope binds to prekallikrein-H-kininogen complex

and activates release of bradykinin. In another embodiment, the method comprises (a) incu-
bating a prekallikrein-H-kininogen complex in the presence of HBP and in the presence and
absence of a monoclonal antibody suspected of binding to at least one epitope on HBP, par-
ticularly human HBP, wherein said epitope binds to prekallikrein-H-kininogen complex and
5   activates release of bradykinin and (b) detecting any effect of said antibody on release of
bradykinin, decreased bradykinin release indicating that said antibody binds to at least said
epitope on HBP.

The invention is also directed to methods and kits for determining if a mammal produces
10   HBP that binds to a HBP antagonist. The method comprises the following steps: (a) isolating
HBP or cells or tissue producing HBP from a mammal, particularly a human patient; (b) incu-
bating said HBP or cells or tissue producing HBP with a substance, tissue, cells or compo-
nent thereof which interacts with HBP and said HBP antagonist and (c) detecting an effect of
said heparin-binding antagonist on the interaction of HBP with said substance, tissue, cells or
15   components thereof, decreased interaction indicating that said HBP binds to said HBP an-
tagonist. The test kit comprises (a) HBP antagonist; (b) native HBP and (c) a substance, tis-
sue, cells or component thereof which interact with HBP.

In a specific embodiment, the invention is also directed to methods and kits for determining if
20   a mammal, particularly a human patient, produces HBP that binds to a monoclonal antibody
that binds to at least on epitope on native HBP, particularly, human HBP, wherein said epi-
tope binds to prekallikrein-H-kininogen complex and activates release of bradykinin, compris-
ing (a) isolating HBP or cells or tissue producing HBP from said mammal; (b) culturing said
HBP or cells or tissue producing HBP with endothelial cells in the presence or absence of
25   said antibody and (c) detecting any effect of said antibody on permeability of endothelial
cells, decreased permeability indicating that said HBP binds to said antibody. In another
embodiment, the method comprises (a) isolating HBP or cells or tissue producing HBP from
said mammal; (b) incubating said HBP or cells or tissue producing HBP with prekallikrein-
kininogen complex in the presence and absence of said antibody and (c) detecting any effect
30   of said HBP on the release of bradykinin from the prekallikrein-H-kininogen complex, de-
creased release of bradykinin indicating that said HBP binds to said antibody. The kit of the
present invention comprises (a) a monoclonal antibody that binds to at least on epitope on
native HBP, particularly, human HBP, wherein said epitope binds to prekallikrein-H-kininogen

complex and activates release of bradykinin, (b) native human HBP and (c) a prekallikrein-H-kininogen complex attached to a solid support.

BRIEF DESCRIPTION OF THE DRAWINGS

5

Figure 1 shows the effect of CD18 crosslinking with secondary goat anti-mouse F(ab)$_2$.

Figure 2 shows the dose-dependent effect of HBP (25-75 µg/ml) on permeability of endothelial cell monolayers.

10

Figure 3 shows the inhibition of HBP-induced increase in EC permeability with polyclonal antiserum to HBP.

Figure 4 shows the effect of anti-HBP antibodies on the actions of PMN secretions on endo-
15 thelial barrier function.

Figure 5 shows inhibition of bradykinin- and HBP-induced increase in EC permeability with monoclonal antibody MBK3 to bradykinin. Bradykinin (100 nM; Figure 5a) or HBP (75 µg/ml; Figure 5b) is administered at time zero to the lamina side of EC monolayers pre-incubated
20 with mAb MBK3 (40 µg/ml).

Figure 6 shows inhibition of HBP-induced increase in EC permeability with monoclonal antibody PKH4 to plasma kallikrein. HBP (75 µg/ml; filled symbols) or bradykinin (100 nM; open symbols).

25

Figure 7 shows inhibition of HBP-induced increase in EC permeability by peptide HKH20-treatment.

Figure 8 shows inhibition of HBP-induced increase in EC permeability by peptide SDD31-
30 treatment.

Figure 9 shows inhibition of HBP-induced increase in EC permeability by aprotinin-treatment.

Figure 10: Il-6 release from human monocytes. The monocytes are cultured in 1 ml serum-free

medium for 24 hours in the presence of LPS and/or native HBP / [R23S,F25E]HBP / [G175Q]HBP in the amounts indicated in the Figure.

Figure 11: Saturation of native HBP, [R23S,F25E]HBP, and [G175Q]HBP with (A) ^{3}H-LPS and
5   (B) Competition for ^{125}I-BPTI binding with fixed, increasing concentrations of unlabeled BPTI. (C) Concentrations of ^{125}I-BPTI shown in % 0 nM of unlabeled BPTI. The apparent difference in binding between HBP and [R23S,F25E]HBP is discussed in the "Results" section. Bars indicate standard deviations.

10   **DETAILED DESCRIPTION OF THE INVENTION**

Heparin-Binding Protein
In the present context, the term "heparin-binding protein" ("HBP"), refers to a protein that is (i) proteolytically inactive; (ii) stored in the azurophil granules of polymorphonuclear leuko-
15   cytes; and (iii) a chemoattractant for monocytes and/or activates monocytes. The HBP may suitably be of mammalian, in particular human origin. In particular, the HBP is a mature human HBP which has at least about an 80% identity with the amino acid sequence set forth in SEQ ID NO:1, more preferably at least about 90%, even more preferably at least about 95%, and most preferably at least about 97% (hereinafter "homologous polypeptides"), which qua-
20   litative retain the activity of said HBP.

SEQ ID NO:1:
Ile Val Gly Gly Arg Lys Ala Arg Pro Arg Gln Phe Pro Phe Leu Ala Ser Ile Gln Asn Gln Gly Arg His Phe Cys Gly Gly Ala Leu Ile His Ala Arg Phe Val Met Thr Ala Ala Ser Cys Phe Gln Ser
25   Gln Asn Pro Gly Val Ser Thr Val Val Leu Gly Ala Tyr Asp Leu Arg Arg Arg Glu Arg Gln Ser Arg Gln Thr Phe Ser Ile Ser Ser Met Ser Glu Asn Gly Tyr Asp Pro Gln Gln Asn Leu Asn Asp Leu Met Leu Leu Gln Leu Asp Arg Glu Ala Asn Leu Thr Ser Ser Val Thr Ile Leu Pro Leu Pro Leu Gln Asn Ala Thr Val Glu Ala Gly Thr Arg Cys Gln Val Ala Gly Trp Gly Ser Gln Arg Ser Gly Gly Arg Leu Ser Arg Phe Pro Arg Phe Val Asn Val Thr Val Thr Pro Glu Asp Gln Cys Arg
30   Pro Asn Asn Val Cys Thr Gly Val Leu Thr Arg Arg Gly Gly Ile Cys Asn Gly Asp Gly Gly Thr Pro Leu Val Cys Glu Gly Leu Ala His Gly Val Ala Ser Phe Ser Leu Gly Pro Cys Gly Arg Gly Pro Asp Phe Phe Thr Arg Val Ala Leu Phe Arg Asp Trp Ile Asp Gly Val Leu Asn Asn Pro Gly Pro Gly Pro Ala

The amino acid sequences of the homologous polypeptides differ from the amino acid se-
quence set forth in SEQ ID NO: 1 by an insertion or deletion of one or more amino acid resi-
dues and/or the substitution of one or more amino acid residues by different amino acid resi-
dues. Preferably, amino acid changes are of a minor nature, that is, conservative amino acid

5   substitutions that do not significantly affect the folding and/or activity of the protein; small de-
letions, typically of one to about 30 amino acids; small amino- or carboxyl-terminal exten-
sions, such as an amino-terminal methionine residue; a small linker peptide of up to about
20-25 residues; or a small extension that facilitates purification by changing net charge or
another function, such as a poly-histidine tract, an antigenic epitope or a binding domain.

10

Examples of conservative substitutions are within the group of basic amino acids (such as
arginine, lysine and histidine), acidic amino acids (such as glutamic acid and aspartic acid),
polar amino acids (such as glutamine and asparagine), hydrophobic amino acids (such as
leucine, isoleucine and valine), aromatic amino acids (such as phenylalanine, tryptophan and

15   tyrosine) and small amino acids (such as glycine, alanine, serine, threonine and methionine).
Amino acid substitutions which do not generally alter the specific activity are known in the art
and are described, e.g., by H. Neurath and R.L. Hill, 1979, in, The Proteins, Academic Press,
New York. The most commonly occurring exchanges are: Ala/Ser, Val/Ile, Asp/Glu, Thr/Ser,
Ala/Gly, Ala/Thr, Ser/Asn, Ala/Val, Ser/Gly, Tyr/Phe, Ala/Pro, Lys/Arg, Asp/Asn, Leu/Ile,

20   Leu/Val, Ala/Glu, Asp/Gly as well as these in reverse.

The term is specifically intended to include peptide fragments of HBP, in particular fragments
with a similar chemotactic effect as native HBP. Furthermore, the HBPs used in the methods
of the present invention bind to HBP antagonists such as aprotinin and/or a monoclonal anti-

25   body generated against native human HBP. Native human HBP in its mature form has the
amino acid sequence shown in SEQ ID NO:1 and additionally is (i) proteolytically inactive;
(ii) stored in the azurophil granules of polymorphonuclear leukocytes; and (iii) a chemoattrac-
tant for monocytes and/or activates monocytes.

30   HBP can be isolated from blood platelets, obtained from human blood using the procedures
described in U.S. Patent No. 5,814,602. More particularly, the protein is produced by frac-
tionation of a blood platelet extract. Column chromatography using heparin-Sepharose is
expedient for this purpose. Such a chromatographic method comprising gradient elution with
NaCl from 0.5 M up to 3 M of a column through which an extract of the blood platelets has

been poured first, results in elution of two peaks. The first peak around 1.2 M NaCl can be measured at 280 nm as a large protein peak and is a platelet factor (PF$_4$) known per se. Around 1.8 M NaCl, the protein amount is below the detection limit in the system used, but the fractions in this area have angiogenic activity. The active fractions are purified addition-

5   ally by microbore reverse phase HPLC on C$_4$ column, and at 214 nm a completely pure protein peak can be detected, and this protein is identical with the present HBP's of either porcine or human type depending on the kind of platelets used.

If the HBP is to be used to detect HBP antagonists, it should preferably be obtained via re-

10   combinant DNA methods as follows. A nucleic acid sequence encoding HBP may be prepared synthetically by established standard methods, e.g., the phosphoamidite method described by S.L. Beaucage and M.H. Caruthers, 1981, Tetrahedron Letters 22: 1859-1869, or the method described by Matthes et al., 1984, EMBO Journal 3: 801-805. According to the phosphoamidite method, oligonucleotides are synthesized, e.g., in an automatic DNA syn-

15   thesizer, purified, annealed, ligated and cloned in suitable vectors.

The techniques used to isolate or clone a nucleic acid sequence encoding the heparin binding protein used in the method of the present invention are known in the art and include isolation from genomic DNA, preparation from cDNA, or a combination thereof. The cloning

20   of the nucleic acid sequences of the present invention from such genomic DNA can be effected, e.g., by using the well known polymerase chain reaction (PCR) or antibody screening of expression libraries to detect cloned DNA fragments with shared structural features. See, e.g., Innis et al., 1990, A Guide to Methods and Application, Academic Press, New York. Other nucleic acid amplification procedures such as ligase chain reaction (LCR),

25   ligated activated transcription (LAT) and nucleic acid sequence-based amplification (NASBA) may be used.

The nucleic acid sequence is then inserted into a recombinant expression vector that may be any vector that may conveniently be subjected to recombinant DNA procedures. The choice of vector will often depend on the host cell into which it is to be introduced. Thus, the vector

30   may be an autonomously replicating vector, i.e., a vector which exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication, e.g., a plasmid. Alternatively, the vector may be one which, when introduced into a host cell, is integrated into the host cell genome and replicated together with the chromosome(s) into which it has been integrated.

The nucleic acid sequence encoding SEQ ID NOS: 1, SEQ ID NO:2, may be operably linked to a nucleic acid encoding a heterologous pro and/or a signal sequence. Alternatively, the nucleic acid encoding SEQ ID NOS:4 (signal sequence + mature HBP) and 6 (signal se-
5   quence + pro sequence + mature HBP), SEQ ID NOS:3 and 5 respectively, may be inserted into a recombinant vector.

SEQ ID NO:2:
atcgttggcg gccggaaggc gaggccccgc cagttcccgt tcctggcctc cattcagaat caaggcaggc acttctgcgg
10   gggtgccctg atccatgccc gcttcgtgat gaccgcggcc agctgcttcc aaagccagaa ccccgggggtt agcaccgtgg
tgctgggtgc ctatgacctg aggcggcggg agaggcagtc ccgccagacg ttttccatca gcagcatgag cga-
gaatggc tacgacccc agcagaacct gaacgacctg atgctgcttc agctggaccg tgaggccaac
ctcaccagca gcgtgacgat actgccactg cctctgcaga acgccacggt ggaagccggc accagatgcc
aggtggccgg ctgggggagc cagcgcagtg gggggcgtct ctcccgtttt cccaggttcg tcaacgtgac tgtgaccccc
15   gaggaccagt gtcgccccaa caacgtgtgc accggtgtgc tcacccgccg cggtggcatc tgcaatggggg ac-
gggggcac cccctcgtc tgcgagggcc tggcccacgg cgtggcctcc ttttcctgg ggccctgtgg ccgaggccct
gacttcttca cccgagtggc gctcttccga gactggatcg atggcgtttt aaacaatccg ggaccggggc cagcctag

SEQ ID NO:3:
20   ggctccagcc ccctttttgga catcgttggc ggccggaagg cgaggccccg ccagttcccg tcctggcct ccattcagaa
tcaaggcagg cacttctgcg ggggtgccct gatccatgcc cgcttcgtga tgaccgcggc cagctgcttc caaagccaga
accccggggt tagcaccgtg gtgctgggtg cctatgacct gaggcggcgg gagaggcagt cccgccagac gttttccatc
agcagcatga gcgagaatgg ctacgacccc cagcagaacc tgaacgacct gatgctgctt
cagctggacc gtgaggccaa cctcaccagc agcgtgacga tactgccact gcctctgcag aacgccacgg
25   tggaagccgg caccagatgc caggtggccg gctggggggag ccagcgcagt gggggggcgtc tctcccgttt
tcccaggttc gtcaacgtga ctgtgacccc cgaggaccag tgtcgcccca acaacgtgtg caccggtgtg ctcacccgcc
gcggtggcat ctgcaatggg gacggggggca ccccctcgt ctgcgagggc ctggcccacg cgtggcctc ctttccctg
gggccctgtg ccgaggccc tgacttcttc acccgagtgg cgctcttccg agactggatc gatggcgttt taaacaatcc
gggaccgggg ccagcctag
30

SEQ ID NO:4
Gly Ser Ser Pro Leu Leu Asp Ile Val Gly Gly Arg Lys Ala Arg Pro Arg Gln Phe Pro Phe Leu
Ala Ser Ile Gln Asn Gln Gly Arg His Phe Cys Gly Gly Ala Leu Ile His Ala Arg Phe Val Met Thr
Ala Ala Ser Cys Phe Gln Ser Gln Asn Pro Gly Val Ser Thr Val Val Leu Gly Ala Tyr Asp Leu
35   Arg Arg Arg Glu Arg Gln Ser Arg Gln Thr Phe Ser Ile Ser Ser Met Ser Glu Asn Gly Tyr Asp

Pro Gln Gln Asn Leu Asn Asp Leu Met Leu Leu Gln Leu Asp Arg Glu Ala Asn Leu Thr Ser
Ser Val Thr Ile Leu Pro Leu Pro Leu Gln Asn Ala Thr Val Glu Ala Gly Thr Arg Cys Gln Val Ala
Gly Trp Gly Ser Gln Arg Ser Gly Gly Arg Leu Ser Arg Phe Pro Arg Phe Val Asn Val Thr Val
Thr Pro Glu Asp Gln Cys Arg Pro Asn Asn Val Cys Thr Gly Val Leu Thr Arg Arg Gly Gly Ile
5 Cys Asn Gly Asp Gly Gly Thr Pro Leu Val Cys Glu Gly Leu Ala His Gly Val Ala Ser Phe Ser
Leu Gly Pro Cys Gly Arg Gly Pro Asp Phe Phe Thr Arg Val Ala Leu Phe Arg Asp Trp Ile Asp
Gly Val Leu Asn Asn Pro Gly Pro Gly Pro Ala

SEQ ID NO:5

10 atgacccggc tgacagtcct ggccctgctg gctggtctgc tggcgtcctc gagggccggc tccagccccc ttttggacat
cgttggcggc cggaaggcga ggccccgcca gttcccgttc ctggcctcca ttcagaatca aggcaggcac ttctgcgggg
gtgccctgat ccatgcccgc ttcgtgatga ccgcggccag ctgcttccaa agccagaacc ccgggggttag caccgtggtg
ctgggtgcct atgacctgag gcggcgggag aggcagtccc gccagacgtt ttccatcagc   agcatgagcg
agaatggcta cgaccccag cagaacctga cgacctgat gctgcttcag ctggaccgtg aggccaacct caccag-
15 cagc gtgacgatac tgccactgcc tctgcagaac gccacggtgg aagccggcac cagatgccag gtggccggct
gggggagcca gcgcagtggg gggcgtctct cccgtttttcc caggttcgtc aacgtgactg tgaccccga ggaccagtgt
cgccccaaca cgtgtgcac cggtgtgctc acccgccgcg gtggcatctg caatggggac   gggggcacc
ccctcgtctg cgagggcctg gcccacggcg tggcctcctt ttccctgggg ccctgtggcc gaggccctga cttcttcacc
cgagtggcgc tcttccgaga ctggatcgat ggcgttttaa acaatccggg accgggggcca gcctag

20

SEQ ID NO:6

Met Thr Arg Leu Thr Val Leu Ala Leu Leu Ala Gly Leu Leu Ala Ser Ser Arg Ala Gly Ser Ser
Pro Leu Leu Asp Ile Val Gly Gly Arg Lys Ala Arg Pro Arg Gln Phe Pro Phe Leu Ala Ser Ile
25 Gln Asn Gln Gly Arg His Phe Cys Gly Gly Ala Leu Ile His Ala Arg Phe Val Met Thr Ala Ala
Ser Cys Phe Gln Ser Gln Asn Pro Gly Val Ser Thr Val Val Leu Gly Ala Tyr Asp Leu Arg Arg
Arg Glu Arg Gln Ser Arg Gln Thr Phe Ser Ile Ser Ser Met Ser Glu Asn GlyTyr Asp Pro Gln
Gln Asn Leu Asn Asp Leu Met Leu Leu Gln Leu Asp Arg Glu Ala Asn Leu Thr Ser Ser Val
Thr Ile Leu Pro Leu Pro Leu Gln Asn Ala Thr Val Glu Ala Gly Thr Arg Cys Gln Val Ala Gly
30 TrpGly Ser Gln Arg Ser Gly Gly Arg Leu Ser Arg Phe Pro Arg Phe Val Asn Val Thr Val Thr
Pro Glu Asp Gln Cys Arg Pro Asn Asn Val Cys Thr Gly Val Leu Thr Arg Arg Gly Gly Ile Cys
Asn Gly Asp Gly Gly Thr Pro Leu Val Cys Glu Gly Leu Ala His Gly Val Ala Ser Phe Ser Leu
Gly Pro Cys Gly Arg Gly Pro Asp Phe Phe Thr Arg Val Ala Leu Phe Arg Asp Trp Ile Asp Gly
Val Leu Asn Asn Pro Gly Pro Gly Pro Ala

The nucleic acid sequence encoding HBP may also be operably connected to a suitable terminator, such as the human growth hormone terminator (Palmiter et al., op. cit.)

The vector may further comprise other elements. These include polyadenylation signals (e.g.
5   from SV 40 or the adenovirus 5 Elb region), transcriptional enhancer sequences (e.g. the SV 40 enhancer) and translational enhancer sequences (e.g. the ones encoding adenovirus VA RNAs). The recombinant expression vector may also comprise a DNA sequence enabling the vector to replicate in the host cell in question. The vector may also comprise a selectable marker, e.g., a gene the product of which complements a defect in the host cell, such as the
10   gene coding for dihydrofolate reductase (DHFR) or one which confers resistance to a drug, e.g., neomycin, geneticin, ampicillin, or hygromycin.

In a specific embodiment, HBP may be produced by culturing host cells containing a DNA sequence encoding mature HBP preceded by an N-terminal extension are in a suitable cul-
15   ture medium under conditions permitting expression of HBP, and recovering the resulting HBP from the culture medium as N-terminally extended HBP.

The N-terminal extension may be a sequence of from about 5 to about 25 amino acid residues, in particular from about 8 to about 15 amino acid residues. The nature of the amino
20   acid residues in the N-terminal sequence is not believed to be critical.

In order to facilitate production of mature HBP, it is generally preferred that the DNA sequence encoding N-terminally extended HBP includes a DNA sequence encoding a protease cleavage site located between the DNA sequence encoding the N-terminal extension and the
25   DNA sequence encoding mature HBP. Examples of suitable protease cleavage sites are an enterokinase cleavage site with the amino acid sequence, a Factor Xa cleavage site with the amino acid sequence.

Alternatively, host cells containing a DNA encoding mature HBP preceded by a signal se-
30   quence may be cultured in a suitable culture medium under conditions permitting expression of HBP and the resulting HBP is recovered from the culture medium as mature HBP.

The procedures used to ligate the nucleic acid sequences coding for HBP or the N-terminally extended HBP, Δpro-HBP (signal + mature HBP), the promoter and the terminator, respec-

tively, and to insert them into suitable vectors containing the information necessary for replication, are well known to persons skilled in the art (cf., for instance, Sambrook et al., op.cit.).

The host cell(s) into which the expression vector is introduced may be any cell(s) which is
5   capable of producing HBP and is preferably a eukaryotic cell(s), such as invertebrate (insect) cell(s) or vertebrate cell(s), e.g., mammalian cell(s), in particular insect and mammalian cell(s). In a preferred embodiment, the mammalian cell is a cell which can be cultured under anaerobic conditions after transfection with a nucleic acid encoding mammalian HBP. In a more preferred embodiment, the mammalian cell is an adenovirus-transformed cell(s) or de
10   rived from an embryonic cell(s). As defined herein, a cell derived from an embryonic cell is a cell(s) obtained from a primary culture of embryonic cells or a cell(s) from a cell line originally passaged from a primary culture of embryonic cells. An example of such an adenovirus-transformed cell or embryonically-derived cell is a human embryonic kidney (HEK) cell(s), particularly HEK 293 cells. The insect cell may, for example, be Lepidoptera or Drosophila
15   cells.

Methods for transfecting mammalian cells and expressing DNA sequences introduced in the cells are described in e.g., Kaufman and Sharp, 1982, J. Mol. Biol. 159:601-621; Southern and Berg, 1982, J. Mol. Appl. Genet. 1:327-341; Loyter et al., 1982, Proc. Natl. Acad. Sci.
20   USA 79:422-426; Wigler et al., 1978, Cell 14:725; Corsaro and Pearson, 1981, Somatic Cell Genetics 7:603, Graham and van der Eb, 1973, Virology 52:456; Fraley et al., 1980, JBC 225:10431; Capecchi, 1980, Cell 22:479; Wiberg et al., 1983, NAR 11:7287; and Neumann et al., 1982, EMBO J. 1:841-845. Insect cells may suitably be transfected with a baculovirus vector as described in U.S. Patent No. 4,745,051.

25

The medium used to culture the cells may be any conventional medium suitable for growing mammalian cells, such as a serum-containing or serum-free medium containing appropriate supplements, or a suitable medium for growing mammalian cells. Suitable media are available from commercial suppliers or may be prepared according to published recipes (e.g. in
30   catalogues of the American Type Culture Collection). The cells are then screened for antibiotic resistance. Subsequently, the selected clones are subsequently assayed for HBP activity using assays known in the art such as a chemotaxis assay and assaying for cytokine release from monocytes (see, for example, Rasmussen et al., 1996, FEBS Lett. 390:109-112).

The HBP produced by the cells may then be recovered from the culture medium by conventional procedures including separating the host cells from the medium by centrifugation or filtration, precipitating the proteinaceous components of the supernatant or filtrate by means

5   of a salt, e.g., ammonium sulphate, purification by a variety of chromatographic procedures, e.g., ion exchange chromatography, affinity chromatography, or the like.

If the HBP is N-terminally extended HBP, after recovery from the culture medium, the N-terminally extended HBP may advantageously be cleaved with a suitable protease to pro-

10   duce mature (and active) HBP. Examples of suitable enzymes include but are not limited to enterokinase and Factor Xa.

HBP ANTAGONISTS

15   An HBP antagonist may be either anti-HBP polyclonal or monoclonal antibodies. The following procedure may be used to obtain anti-HBP polyclonal antibodies. For the production of antibody, various host animals can be immunized by injection with the HBP, preferably human HBP, including but not limited to rabbits, mice, rats, sheep, goats, etc. In one embodiment, HBP can be conjugated to an immunogenic carrier, e.g., bovine serum albumin (BSA)

20   or keyhole limpet hemocyanin (KLH). Various adjuvants may be used to increase the immunological response, depending on the host species, including but not limited to Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanins, dinitrophenol, and potentially useful human adjuvants such as BCG

25   (bacille Calmette-Guerin) and Corynebacterium parvum.

The following procedure may be used to obtain monoclonal antibodies that bind to at least one epitope of HBP, in particular, human HBP, wherein said epitope binds to prekallikrein-H-kininogen complex and activates release of bradykinin. In a preferred embodiment, the mo-

30   noclonal antibody is a human monoclonal antibody. Any technique that provides for the production of antibody molecules by continuous cell lines in culture may be used. These include but are not limited to the hybridoma technique originally developed by Kohler and Milstein (1975, Nature 256:495-497), as well as the trioma technique, the human B-cell hybridoma technique (Kozbor et al., 1983, Immunology Today 4:72), and the EBV-hybridoma

technique to produce human monoclonal antibodies (Cole et al., 1985, in Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc., pp. 77-96). In an additional embodiment of the invention, monoclonal antibodies can be produced in transgenic animals utilizing recent technology (PCT/US90/02545; Green, 1999, J. Immunol. Methods 231:11-23 and U.S. Pat.

5   Nos. 5,625,126 and 5,633,425). According to the invention, human antibodies may be used and can be obtained by using human hybridomas (Cote et al., 1983, Proc. Natl. Acad. Sci. U.S.A. 80:2026-2030) or by transforming human B cells with EBV virus in vitro (Cole et al., 1985, in Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, pp. 77-96).

10   According to the invention, techniques described for the production of single chain antibodies (U.S. Pat. No. 4,946,778) can be adapted to produce HBP-specific single chain antibodies. An additional embodiment of the invention utilizes the techniques described for the construction of Fab expression libraries (Huse et al., 1989, Science 246:1275-1281) to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity for HBP.

15

Antibody fragments which contain the idiotype of the antibody molecule can be generated by known techniques. For example, such fragments include but are not limited to: the F(ab').sub.2 fragment which can be produced by pepsin digestion of the antibody molecule; the Fab' fragments which can be generated by reducing the disulfide bridges of the

20   F(ab').sub.2, fragment, and the Fab fragments which can be generated by treating the antibody molecule with papain and a reducing agent.

In the production of antibodies, screening for the desired antibody can be accomplished by techniques known in the art, e.g., radioimmunoassay, ELISA

25   (enzyme-linked immunosorbent assay), "sandwich" immunoassays, immunoradiometric assays, gel diffusion precipitin reactions, immunodiffusion assays, in situ immunoassays (using colloidal gold, enzyme or radioisotope labels, for example), western blots, precipitation reactions, agglutination assays (e.g., gel agglutination assays, hemagglutination assays), complement fixation assays, immunofluorescence assays, protein A assays, and immunoelectro-

30   phoresis assays, etc. In one embodiment, antibody binding is detected by detecting a label on the primary antibody. In another embodiment, the primary antibody is detected by detecting binding of a secondary antibody or reagent to the primary antibody. In a further embodiment, the secondary antibody is labeled. Many means are known in the art for detecting binding in an immunoassay and are within the scope of the present invention.

Alternatively, a library of anti-HBP antibodies can be generated using phage-display tech-niques (see, for example, Koscielska et al., 1998, Acta Biochimica Polinica 45:705-720). The library may be screened and the strongest binders may be used as HBP antagonists.

5

The HBP antagonist may also be a polypeptide comprising a Kunitz-type serine protease in-hibitor domain that binds to HBP. Peptide comprising Kunitz-type serine protease inhibitor domains generally contain about 60 residues and six specifically spaced cysteines that are present in disulfide bonds. In a specific embodiment, if the HBP is human HBP, the Kunitz-

10   type inhibitor may be aprotinin also known as BPTI or analog thereof that binds to HBP. In a preferred embodiment, the aprotinin analog has mutations at the 12-19 and 34-39 positions, especially preferred are mutations at the 15-19 positions. Other analogs that may be used are disclosed in U.S. Patent Nos. 5,162,498, 5,316,923, 5,395,922, 5,514,585, 5,510,249, 5,591,603, 5,618,915, 5,621,074, 5,673,090, 5,618,696 and 5,576,294. In a most specific

15   embodiment, the mutations are A16H, K15A, I18M, I19S, R17T.

Large libraries (10^7-10^9) of polypeptides comprising kunitz-type domains may be obtained using a variety of approaches. For example, error-prone PCR (Saiki et al., 1988, Science 293:487-491) may be applied to each individual kunitz-type domain. This procedure will ran-

20   domly incorporate the incorrect nucleotide in the gene for each corresponding kunitz-type domain by running the PCR reaction under conditions where the Taq polymerase incorpo-rates wrong nucleotides in the amplification step. The library may be displayed on phage particles to select the best binder to HBP.

25   Alternatively, specific loops may be subjected to mutagenesis by the use of degenerate oli-gonucleotides (Schier et al., 1996, Gene 169:147-153). Synthetic random oligonucleotides are incorporated into the kunitz-type domain during the PCR reaction generating a library with variants in a particular loop.

30   In yet another approach, libraries may be generated via DNA shuffling (Stemmer et al., 1994, Nature 370:389-391). This method can be used for recombination between homologous ku-nitz-type domains. In short, DNA coding for each variant is mixed and fragmented to small pieces. These pieces are then reassembled in a regular PCR reaction. During this reaction,

the small pieces will join each other because they are complementary to each other leading recombination of the genes included.

Detection Methods

5 Heparin-binding protein (HBP) antagonists may be detected by determining if a substance suspected of being a HBP antagonist binds to HBP by (a) incubating HBP with a first substance that interacts with HBP and a second substance suspected of being a HBP antagonist and (b) detecting any effect of said second substance suspected of being an antagonist on interaction of HBP with said first substance, wherein decreased interaction of HBP with first

10 substance is indicative that second substance is an HBP antagonist.

The HBP or first substance may be labeled. The label is preferably selected from the group consisting of enzymes, colored or fluorescent substances, radioactive isotopes and complexing agents.

15

Examples of enzymes useful as labeled substances re peroxidases (such as horseradish peroxidase), phosphatases (such as acid or alkaline phosphatase), B-galactosidase, urease, glucose oxidase, carbonic anhydrase, acetylcholinersterase, glucoamylase, lysozyme, malate dehydrogenase, glucose-6-phosphate dehydrogenase, b-glucosidase, proteases, py-

20 ruvate decarboxylase, esterases, luciferase, etc.

Enzymes are not in themselves detectable but must be combined with a substrate to catalyze a reaction, the end product of which is detectable. Examples of substrates which may be employed in the method according to the invention include hydrogen perox-

25 ide/tetramethylbenzidine or chloronaphthole or o-phehylenediamine or 3-(p-hydroxyphenyl) propionic acid or luminol, indoxyl phosphate, p-nitrophenylphosphate, nitophenyl galactose, 4-methyl umbelliferyl-D-galactopyranoside or luciferin.

Alternatively, the labeled substance may comprise colored or fluorescent substances, includ-

30 ing gold particles, colored or fluorescent latex particles, dye particles, fluorescein, phyco-erythrin or phycocyanin.

Radioactive isotopes which may be used for the present purpose may be selected from ^{125}I, ^{131}I, ^{111}In, ^{3}H, ^{32}P, ^{14}C and ^{35}S. The radioactivity emitted by these isotopes may be measured in a gamma-counter or a scintillation counter in a manner known in the art.

5   Complexing agents which may be employed for the present purpose may be selected from biotin (which complexes with avidin or streptavidin), avidin (which complexes with biotin), Protein A (which complexes with immunoglobulins) and lectins (complexing with carbohydrate receptors). Since the complex is not directly detectable, it is necessary to label the substance with which the complexing agent forms a complex. The labelling may be carried

10   out with any one of the labeled substances mentioned above fro the labelling of the enzyme.

In a specific embodiment, the binding of HBP antagonist to HBP may be detected by using, for example, SPA technology from Amersham Pharmacia Biotech. Briefly, biotinylated plasma membranes from umbilical vein endothelial cells (HUVEC) are bound to streptavidin

15   conjugated PVT SPA beads. These beads may be obtained from Amersham Pharmacia Biotech. Labeled human kininogen (for example, ^{3}H, ^{125}I) is added to the membranes until the binding sites for kininogen are saturated. HBP is added in increasing concentrations to displace the labeled kininogen and the decline in incorporated label is measured. A standard displacement curve is prepared. HBP antagonist is tested for its ability to inhibit HBP medi-

20   ated displacement of radioactive kininogen for the plasma membranes. Specifically, HBP is incubated with various concentrations up to molar excess of HBP antagonists and their ability to inhibit HBP mediated displacement of the labeled kininogen from the membranes is determined.

25   Alternatively, HBP antagonists may be detected by culturing endothelial cells in the presence of HBP and in the presence and absence of a substance suspected of being an antagonist. In a specific embodiment, the antagonist is a monoclonal antibody that binds to at least one epitope of HBP, preferably human HBP, wherein said epitope binds to prekallikrein-H-kininogen complex and activates release of bradykinin. In a preferred embodiment, the mo-

30   noclonal antibody is a human monoclonal antibody. In one embodiment, the antibody and HBP are preincubated together before incubating with endothelial cells for about 10 minutes at 37°C. The effect of the substance on the permeability of endothelial cells is determined by measuring transendothelial electrical resistance or determining albumin clearance as de-

scribed in the Examples, *infra*. A decrease in permeability would indicate that the substance does act as an HBP antagonist.

In a specific embodiment, a monoclonal antibody binding to at least one epitope of HBP,
5   wherein said epitope binds to prekallikrein-H-kininogen complex and activates release of bradykinin, can be detected by incubating the antibody with HBP and a prekallikrein-H-kininogen complex. As will be described in further detail in the Examples, HBP binds to the prekallikrein-H-kininogen complex thereby stimulating the release of bradykinin. A monoclonal antibody that binds to at least one epitope of HBP would decrease or prevent the re-
10 lease of bradykinin. The release of bradykinin may be assayed using procedures known in the art, e.g., immunoassay.

In one embodiment, coating buffer containing 0.05% Tween, 15.9 mM disodium carbonate 35 mM sodium hydrogen carbonate, pH 9.6. H-kininogen is added and incubated at 4°C
15 overnight. The coating buffer is removed and the plate is washed with Tris buffer, pH 7.4 containing about 50 M zinc. Prekallikrein is then added and the mixture is incubated for 1 hr. at room temperature. HBP in Tris buffer is subsequently added and samples are removed at about 10 minute intervals for about 1 hour beginning about 5 minutes after adding HBP and assayed for bradykinin release. In one embodiment, bradykinin release may be
20 assayed using a commercially available ELISA assay (MARKIT-M Bradykin, Dainippon Pharmaceutical Co., Ltd.). In such an assay, bradykinin in a sample and peroxidase labeled bradykinin are allowed to react competitvely with anti-BK antibody (rabbit) captured by anti-rabbit IgG antibody (goat) coated on a microstrip well. The bradykinin concentration is determined from the enzyme activity of peroxidase labeled bradykinin bound to anti-bradykinin
25 antibody.

Alternatively, prekallikrein and H-kininogen may be added in equimolar amounts to a mono-layer of endothelial cells and incubated for 1 hr. at 37°C. HBP is subsequently added and samples are removed at about 10 minute intervals for about 1 hour beginning about 5 minu-
30 tes after adding HBP and assayed for bradykinin release using procedures described above.

In yet another embodiment, prekallikrein and H-kininogen may be added in equimolar amounts to a plasma solution containing ACE (angiotensin converting enzyme) inhibitor and

incubated for 1 hr. at 37°C.   HBP is subsequently added and samples are removed at about 10 minute intervals for about 1 hour beginning about 5 minutes after adding HBP and assayed for bradykinin release using procedures described above.

5   **Treatment Methods**

The composition of the present invention comprises part of an aqueous solution. The solution preferably is physiologically acceptable so that in addition to delivery of the desired construct to the patient, the solution does not otherwise adversely affect the patient's electrolyte and volume balance. The aqueous medium for the composition thus may comprise, for example,

10   normal physiological saline (0.9% NaCl, 0.15M), pH 7-7.4 or other pharmaceutically acceptable salts thereof. . Useful solutions may be prepared by any of the methods well known in the pharmaceutical art, described, for example, in "Remington's Pharmaceutical Sciences", (Gennaro, A., ed.), Mack Pub., 1990.

15   The concentration of HBP antagonist may vary widely, i.e. from less than about 0.5%, such as from 1%, to as much as 15-20% by weight. A unit dosage of the composition may typically contain from about 10 mg to about 1 g of HBP antagonist. Therapeutically effective dosages may be determined by either in vitro or in vivo methods.

20   HBP antagonist is administered topically, subcutaneously or by intravenous injection. Dosages will be prescribed by the physician according to the particular condition and the particular individual to be treated. Dosages and frequency is carefully adapted and adjusted according to parameters determined by the physician in charge. A preferred administration route may be, e.g., injections intraperitoneally. Intravenous intraperitoneal injections of HBP an-

25   tagonist may be given per 24 hours in the range of from 0.1-100 mg, especially 0.5-50 mg, in particular 1-25 mg per kg body weight. The dose may be given 1-4 times per 24 hours or administered continuously through a catheter. Alternatively, treatments may be given via continuous infusion during surgery intravenously, then subsequent treatment for 1-4 days after surgery.

30

The HBP antagonist will be administered only to those patients producing HBP that binds to the HBP antagonist. To determine if the patient produces such HBP, the HBP from the patient will be isolated from the platelets from the blood of patients using procedures described above. The binding of HBP to HBP antagonist (for example, a monoclonal antibody) or ef-

fect of HBP on the effect on endothelial cell permeability may be detected using procedures described above and compared to the effects of native HBP. Alternatively, the effect of HBP on endothelial cell or fibroblast cell contraction or monocyte aggregation in the presence or absence of HBP antagonist may also be determined using the procedures described in

5 WO93/05396. A decreased degree of cell contraction or aggregation indicates the presence of an interaction between HBP and HBP antagonist. A test kit that may be used to determine if a patient produces such HBP comprises comprising (a) HBP antagonist; (b) a HBP or cell producing HBP and (c) a substance, tissue, cells or component thereof which interact with HBP.

10

In a specific embodiment, HBP or cells or tissue producing HBP is isolated from a patient. The HBP is or cells or tissue producing said HBP are incubated with endothelial cells and the effect on permeability is determined in the presence and absence of monoclonal antibody that binds to at least one epitope of HBP, wherein said epitope binds to prekallikrein-H-

15 kininogen complex and activates release of bradykinin. This effect is compared to the effect of native human HBP. In a specific embodiment, the native human HBP may be recombinant HBP which bind to such a monoclonal antibody. Alternatively, HBP or cells or tissue producing HBP are incubated in the presence of prekallikrein-H-kininogen complex and in the presence of monoclonal antibody. The effect of the monoclonal antibody on bradykinin

20 release is determined. A decrease in bradykinin release in the presence of antibody indicates that the patient produces HBP that binds to the antibody. In such a situation, the test kit would comprise (a) a monoclonal antibody that binds to at least one epitope on HBP, wherein said epitope binds to prekallikrein-H-kininogen complex and activates release of bradykinin; (b) native human HBP and (c) prekallikrein-H-kininogen complex attached to a

25 solid support.

EXAMPLES

Example 1: Activated neutrophils trigger alterations in endothelial barrier function through signaling via ß2 integrins

5

MATERIALS AND METHODS

Reagents

Medium-199, RPMI-1640 (containing L-glutamine), fetal bovine serum (FBS), trypsin-EDTA, phosphate buffered saline (PBS) and Hanks balanced salt solution (HBSS) are from Life

10   Technologies (Gaithersburg, MD, USA). Gelatin, collagenase, penicillin, streptomycin, catalase, bovine serum albumin (BSA), N-formyl-methionyl-leucyl-phenylalanine (fMLP), Evans blue dye, hexadecyltrimethylammonium bromide and tetramethylbenzidine are obtained from Sigma Chemical Co. (St. Louis, MO, USA). H_2O_2 is obtained from E. Merck (Darmstadt, Germany). Biomatrix I is obtained from Biomedical Technologies Inc. (Stoughton, MA, USA),

15   and Dextran 70 (Macrodex) and Ficoll-Paque are from Pharmacia Biotech AB (Uppsala, Sweden). The basal culture medium consisted of a 1:1 mixture of M-199 and RPMI-1640 supplemented with 20% heat inactivated FBS, penicillin (100 U/ml) and streptomycin (100µg/ml). The monoclonal antibody IB4 against the common β-chain (CD18) of $β_2$-integrins is provided by Dr. Claes Lundberg, Pharmacia & Upjohn (Uppsala, Sweden) by courtesy of

20   Dr. Samuel D. Wright, Rockefeller University, mAb 60.1 recognizing integrin $α_m$ chain (CD11b) is provided by Dr. Manuel Patarroyo, Karolinska Institutet, mAb DREG 200 (anti-L-selectin) is a gift of Dr. Eugene Butcher, Stanford University, mAb SK11 (anti-L-selectin) is from Becton Dickinson (San José, CA) and mAb G44-26 (anti-CD44) is obtained from PharMingen (San Diego, CA). F(ab')$_2$ fragment of goat anti-mouse IgG is obtained from Jackson

25   ImmunoResearch Laboratories, Inc. (West Grove, PA), FITC-conjugated F(ab')$_2$ fragment of rabbit anti-mouse IgG is from Dako A/S (Glostrup, Denmark).

Endothelial cells

Human umbilical vein endothelial cells (HUVEC) and bovine aorta endothelial cells (BAEC)

30   are isolated and cultured as previously described (Gautam *et al.* 1998, Brit. J. Pharm. 125:1109-1114). HUVEC or BAEC in the first to fifth passages are detached by brief (2 min) trypsin-EDTA treatment (0.25% trypsin/0.01 % EDTA) and replated onto either 0.2 µm pore size Anopore inorganic membrane or 3.0 µm pore size polycarbonate filters (Tissue Culture inserts, 10 mm; NUNC, Roskilde, Denmark). In order to promote cellular differentiation and

enhance attachment of endothelial cells (ECs), the filters are pretreated with 50 µl Biomatrix I
(167 µg/ml) and air-dried. ECs are seeded at a density of 2×10^5 cells per filter and incu-
bated in culture medium at 37°C in a humidified atmosphere of 5% CO_2 in air. The EC are
grown to confluent monolayers, as assessed by daily microscopic observation and meas-
5   urement of the electrical resistance across the monolayer (Gautam et al. 1998, Brit. J.
Pharm. 125:1109-1114).

Measurement of EC barrier function

Two different approaches are used for assessment of stimulation-induced changes in EC
10   permeability. For measurement of transendothelial electrical resistance (TEER), the filter in-
sert with EC is transferred to a resistance measurement chamber (for details, see Gautam et
al. 1998, Brit. J. Pharm. 125:1109-1114). The chamber (lower/luminal compartment) with the
filter insert (upper/luminal compartment), filled with 2 ml and 400µl culture medium respec-
tively is placed in a cell culture incubator. Electrical resistance across the EC monolayer is
15   measured at 37°C with electrodes in each compartment exactly positioned in relation to each
other and to the monolayer. The electrical resistance of individual EC monolayers is obtained
by subtracting the resistance of the corresponding naked filter coated with Biomatrix as mea-
sured prior to seeding of the endothelial cells.

20   In separate experiments, Evans blue dye-conjugated albumin (EBA) is used as a marker for
macromolecular permeability of the EC monolayers. Prior to stimulation of EC, the culture
medium in the upper compartment is exchanged for medium containing EBA (culture me-
dium containing 4% BSA mixed with Evans blue dye at a final concentration of 0.67 mg/ml).
The EBA concentration in fluid samples of the upper and lower compartment is determined
25   spectrophotometrically through measurement of absorbency at 620 nm (Titertek Multiskan
MCC; Flow Laboratories, Solna, Sweden). Albumin clearance is calculated according to the
relationship: $V1 = A2 \times V2 \times 1/A1$, where V1 is the clearance volume (i.e. the theoretical vol-
ume of the luminal medium cleared from albumin by diffusion to the abluminal compartment),
V2 is the abluminal volume, and A1 and A2 is the absorbency of the luminal and abluminal
30   medium, respectively. The baseline clearance in absence of any stimulus, averaging 0.08 ±
0.03 µl/min, is subtracted from the clearance volume obtained in response to respective spe-
cific stimulus.

Preparation and quantification of PMNs

Human PMNs are isolated from leukocyte-rich plasma as described (Gautam et al., 1998, Brit. J. Pharm. 125:1109-1114), and resuspended in culture medium at a concentration of 2-5 x 10^7 cells/ml. The purity of the PMNs is > 98% and their viability by

5   Trypan blue exclusion is >95%. Before being added to the EC monolayers, the PMNs are incubated for 30 min with monoclonal antibodies against cell surface molecules for subsequent cross-linking with secondary mAb (see experimental procedures).

In some experiments, cell surface expression of CD11b/CD18 is upregulated through

10 temperature transition of the PMNs prior to use. PMNs in the presence of anti-CD18 mAb IB4 are incubated at 4°C for 10 min, transferred to 37°C for 10 min and then returned to 4°C for another 10 min followed by two washes. PMNs not subjected to antibody treatment are prepared according to the same protocol using vehicle instead of antibody.

15

PMN adhesion to EC and transendothelial migration are quantified by assaying the PMN-specific enzyme myeloperoxidase (MPO). In brief, PMNs are lysed in 0.5 % hexadecyltrimethylammonium bromide and centrifuged, and the enzyme activity in the supernatant determined spectrophotometrically as the change in absorbency at 650 nm

20 that occurs in the redox reaction of H_2O_2-tetramethylbenzidine catalyzed by MPO (Suzuki et al., 1983, Anal. Biochem. 132:345-352). MPO activity of adherent and transmigrated PMNs, respectively, is related to that of the total number of PMNs added to the EC monolayer.

25 ### Experimental procedures

Before transferring the filter insert to the resistance measurement chamber (kept at 37°C), the medium in the insert and in the chamber is replaced with fresh culture medium (37°C) supplemented with 10 mM Hepes. In some experiments, the EC monolayer is incubated before stimulation with 10 µg/ml Herbimycin A for 15 min at 37°C and washed twice. PMNs

30 (2 x 10^6), with or without monoclonal antibodies bound to cell surface molecules, are added to the upper compartment (PMN:EC ratio = 10:1) and allowed to sediment for 10 min onto the EC monolayer. Activation of PMN is induced either with fMLP (10^{-7} M) added to the lower compartment or through antibody cross-linking of cell surface molecules by secondary goat-anti-mouse IgG F(ab')$_2$ fragments. Transendothelial electrical resistance is measured before

and every minute after start of stimulation until a plateau phase in the resistance change is reached, and then at 5-min intervals. In experiments where albumin permeability and PMN migration across the EC monolayer are studied, the medium in the insert contained EBA. After challenge, the filter insert is moved at 5-10 min intervals to new wells containing fresh

5   medium. All incubations are performed at 37°C. At the end of the experiment, the lower wells are centrifuged at 425g and 4°C for 20 min and the supernatant is analyzed for EBA content. PMN that had migrated across the monolayer are quantified through analysis of the MPO-activity of the pellet remaining in the well. The medium in the filter insert is aspirated for quantification of non-adherent PMN, and the filter membrane with EC is removed from the

10   insert to quantify the adherent fraction of PMN.

In separate experiments, purified PMNs, pretreated with the anti-CD18 mAb IB4 (6 µg per 2×10^6 cells) for 30 min, are incubated in culture medium with or without goat-anti-mouse F(ab')$_2$ for 10 min at 37°C. The PMNs are sedimented by centrifugation at 300xg for 15 min

15   at room temperature and the cell-free supernatant containing PMN secretion products is decanted for use in experiments *in vivo* (see below) or with the EC-monolayers. In this latter setting, the medium in the filter/EC insert is exchanged for the PMN secretion-containing medium, and effects on TEER and albumin permeability are recorded as described above. In some cases, the PMN secretion-containing medium is heat-incubated

20   (80°C for 15 min) before use.

Determination of EC intracellular $[Ca^{2+}]$ and f-actin formation
The Ca^{2+} sensitive fluorescent probe fluo-3/AM (Molecular Probes Europe BV, Leiden, The Netherlands) is used for determination of changes in EC intracellular free Ca^{2+}. Confluent

25   BAEC monolayers on filters are incubated for 30 min at 37°C with fluo-3/AM (3µM in HBSS containing 2% FCS and 10 mM HEPES) added to both the apical and basolateral surface. The monolayers are washed three times and incubated with fresh HBSS in the dark for 20 min at room temperature to allow complete hydrolysis of the dye ester. Anti-CD18 or anti-L-selectin mAb treated PMNs are added to ECs and receptor cross-linking is induced with sec-

30   ondary mAb as described above. In separate experiments, PMN secretion containing medium (see above) is used to stimulate ECs in the absence of PMNs. Changes in EC $[Ca^{2+}]_i$ in response to these stimuli are measured through continuous registration of fluorescence intensity with a laser-scanning confocal imaging system (Insight Plus; Meridian Instruments Inc., Okemon, Michigan).

For analysis of f-actin formation in ECs in response to PMN activation, confluent EC mono-
layers grown on Biomatrix coated coverslips are incubated for 15 min at 37°C either with
PMN subjected to CD18 antibody cross-linking or with PMN secretion containing medium
5   identical to the procedures described above. Incubation of ECs with IB4-treated PMN ser-
ved as control. The monolayers are fixed in 3.7% formaldehyde in PBS for 10 min at room
temperature, washed twice and permeabilized with 0.2% Triton X-100 in PBS (1 min at 4°
C). The cells are washed twice and stained for actin filaments with FITC-conjugated phal-
loidin for 20 min at 37°C. After three additional washes the endothelial cells are viewed in a
10   laser-scanning confocal imaging system (Insight Plus; Meridian Instruments Inc., Okemon,
Michigan).

In vivo experiments

In order to study the effect of PMN secretion products on microvessel permeability in vivo,
15   intravital microscopy of the hamster cheek pouch microcirculation is applied. As previously
described in detail (Raud & Lindbom, 1994, Immunology of the Microcirculation, Academic
Press, 1994, chapter 7), the left cheek pouch of anaesthetized Syrian golden hamsters is
everted and prepared for microscopic observation under continuous superfusion with a bi-
carbonate buffer maintaining physiological levels of temperature, pH and gas tensions.
20   FITC-conjugated dextran (Mw: 150.000) injected i.v. (250 mg/kg body weight) is used as
plasma tracer to permit visualization of changes in vascular permeability to macromolecules.
The microvasculature in the exposed cheek pouch is observed under low magnification in
fluorescent light with a Leitz Orthoplan microscope and recorded on video. After control re-
cordings, superfusion is stopped and 1 ml of medium containing CD18 cross-linking-evoked
25   PMN secretion at 37° C is topically applied to the cheek pouch. In parallel experiments, anti-
CD18-treated PMN medium is not subjected to cross-linking is used.

RESULTS

30   PMNs (2×10^6), pre-treated with anti-CD18 mAb (IB4), are added to the EC/filter insert (lu-
minal side of EC/upper compartment of the resistance chamber) and allowed to settle for 10
min. CD18 cross-linking with secondary goat anti-mouse F(ab)$_2$ elicited a marked decrease
in TEER, which is manifested within 1 min, reached its maximum (36±13 of control) after 15
min and remained at this level throughout the observation period (Figure 1). Neither treat-

ment of PMNs with IB4 alone nor with secondary mAb in the absence of IB4 caused any change in TEER.

The capacity of CD18 cross-linking in causing increased macromolecular flux and PMN mi-
5　gration across the EC monolayer is studied in additional experiments. PMNs pre-treated with saturating concentration of IB4 are added to the EC/filter insert and allowed to settle for 10 min in medium containing EBA. CD18 cross-linking is similarly induced with goat anti-mouse F(ab)$_2$ and the PMN/EBA containing filter inserts are moved at regular intervals to new wells containing fresh medium. In the absence of secondary antibody, there is a minute and con-
10　tinuous clearance of albumin, 0.08±0.03 µl·min^{-1} (mean±SD, n=13), which did not change with time and which exactly matched the spontaneous clearance rate found in filter inserts containing untreated PMNs. By contrast, activation of the PMNs by CD18 cross-linking elic-
ited augmented albumin clearance, which increased progressively with time (Figure 1). The net increase in albumin clearance rose from 0.5±0.1 µl during the first 5-min period to a total
15　of 20.0±4.5 µl (mean±SD, n=10) in 60 min. Distinct from increased albumin clearance, CD18 cross-linking caused no measurable PMN adhesion to EC or transendothelial migration (Fig-
ure 1). Analysis of MPO activity in the different compartments 60 min after CD18 cross-
linking revealed that 91±6 % (mean±SD, n=9) of added PMNs are recovered in medium col-
lected from the upper compartment, whereas no significant MPO-activity is detected in the
20　EC/filter-unit or in the medium of the lower compartment. Direct microscopic observation of transparent filters washed at the end of the experiment confirmed the absence of PMNs ad-
herent to the EC monolayer.

Monolayers of endothelial cells are labelled with the Ca^{2+}-sensitive fluorophore fluo-3, and
25　stimulus-induced changes in EC [Ca^{2+}]$_i$ monitored by confocal laser scanning microscopy. Adding anti-CD18 treated PMNs to the EC monolayer caused no change in EC [Ca^{2+}]. How-
ever, CD18 cross-linking with secondary mAb elicited a rapid increase in EC cytosolic free [Ca^{2+}], which peaks after approximately 100 sec and then subsided slowly. Antibody cross-
linking of L-selectin in an equivalent manner failed to induce any change in EC [Ca^{2+}],
30　whereas a Ca^{2+}-response similar to that induced by CD18 cross-linking is seen after stimula-
tion of untreated PMNs with fMLP (c.f. Gautam et al, 1998, Brit. J. Pharmacol. 125:1109-
1114). A causal link between intracellular Ca^{2+}-activity changes and the functional response of EC to PMN activation is evidenced by experiments where the endothelial cells are pre-
treated with the calcium-chelating agent BAPTA AM. This treatment completely inhibited both

the rise in EC free intracellular Ca^{2+} and the change in TEER in response to CD18 cross-linking.

In further experiments, EC monolayers are incubated with PMNs and then stained for actin
filaments with FITC-conjugated phalloidin. Confocal laser scanning microscopy revealed that
endothelial cells incubated with IB4-treated PMN in absence of secondary antibody exhibited
few stress fibers and a thin band of actin along cell margins in direct apposition with an adja-
cent cell. On the other hand, endothelial cells incubated with PMN subjected to CD18 cross-
linking presented a marked increase in f-actin content and in the number and density of
stress fibers.

To investigate if factor(s) secreted by the PMNs govern the response ultimately leading to
increased EC permeability, PMNs ($2x10^6$), pre-treated with anti-CD18 mAb IB4 and washed
to remove unbound antibody, are incubated with goat anti-mouse $F(ab')_2$ for 30 min and then
spun down by gentle centrifugation. Addition of cell-free supernatant to the EC monolayer
elicited a fall in TEER and an increase in albumin clearance, both of which with the same
magnitude and time course as after CD8 cross-linking in PMNs added to EC. Also, the su-
pernatant caused changes in $EC[Ca^{2+}]_i$ and in f-actin content and distribution. Moreover, to-
pical administration of the PMN secretion-containing supernatant to the hamster cheek
pouch *in vivo*-preparation elicited prompt leakage of plasma from postcapillary and small
venules. Leaky spots are observed already within 1.5 min after application, and a maximal
response manifested after approximately 5 min. After wash of the tissue with buffer the leak-
age slowly subsided. This effect on microvessel permeability is lost after heat-treatment of
the PMN-derived secretion indicating that a heat-sensitive factor(s) is responsible for its per-
meability-increasing activity. Application to the cheek pouch of supernatant of IB4-treated
PMNs not subjected to cross-linking did not cause any visible plasma leakage.

DISCUSSION

Polymorphonuclear leukocyte adhesion to the endothelial lining and recruitment to extravas-
cular tissue in acute inflammation is critically dependent on the function of β2 integrins. In-
creased vascular permeability for macromolecules is induced in conjunction with this cellular
response leading to plasma exudation and edema formation. The results of the above-
described experiments have provided evidence for a causal link between transmembrane
(outside-in) signaling by β2 integrins in activated PMNs and the capacity of these cells to in-

duce EC barrier dysfunction. It is shown that antibody-induced ligation and clustering of β2
integrins triggers an increase in EC permeability similar to that seen after chemoattractant
stimulation of the PMNs. This cellular cross-talk occurs in absence of physical binding of
PMNs to EC indicating that the event of PMN adhesion and structural linkage per se does

5   not directly transfer signals to EC whereby the permeability response is mediated. Rather, β2
integrin ligand-binding initiates through an intracellular signaling cascade PMN secretory
events and release of a chemical messenger, which capacitates the PMN-dependent change
in EC permeability. Further, it is demonstrated that the alteration in vascular permeability
induced via this pathway is a consequence of active, tyrosine kinase-dependent responses

10   of EC involving cytoskeletal reorganization and reversible intercellular gap formation indicat-
ing a non-lytic function in this respect of the PMN-derived factor(s).

In order to explore in more detail mechanisms by which activated PMNs may transfer signals
to ECs in this process, PMN/EC interactions and adhesion-dependent engagement of β_2 in-

15   tegrins are simulated through antibody cross-linking of CD11/CD18. Through antibody cross-
linking of ß2 integrins, bypassing chemoattractant-induced cellular activation, it is demon-
strated that engagement and clustering of ß2 integrin receptors per se via intracellular signal-
ing events trigger an increase in EC permeability. Since this response occurred irrespective
of PMN adhesion to EC, transfer of signals between PMNs and EC cannot be ascribed to an

20   interaction of CD11/CD18 with counter-receptors on the EC surface, but rather, must be ac-
complished via release of a PMN-derived messenger molecule. Such mechanism is further
supported by the capacity to induce EC hyperpermeability of the cell-free supernatant ob-
tained after CD18 cross-linking in PMNs in suspension.

25   **Example 2: Identification of Neutrophil-Derived HBP (HBP) as Signaling Link in Neu-
trophil-Induced Vascular Plasma Leakage**

In Example 1, PMN adhesion through the β-2 integrin is a prerequisite for the PMN induced
effects on endothelial cell permeability and that a Ca^{2+} dependent transmembrane signaling

30   through the adhesion activated β-2 integrin induced secretion of a soluble factor(s) which di-
rectly trigger the changes in the EC barrier function.

32

As described in Example 2 herein, the soluble factor is determined to be human HBP. Example 2 also describes the characterization of the interaction of HBP with the contact phase system.

5   **MATERIALS AND METHODS**

Reagents

Medium-199, RPMI-1640 (containing L-glutamine), fetal bovine serum (FBS), trypsin-EDTA, phosphate buffered saline (PBS) and Hanks balanced salt solution (HBSS) are from Life

10   Technologies (Gaithersburg, MD, USA). Gelatin, collagenase, penicillin, streptomycin, bovine serum albumin (BSA), Evans blue dye and FITC-conjugated phalloidin are obtained from Sigma Chemical Co. (St. Louis, MO, USA). Fluo-3/AM are obtained from Molecular Probes (Eugene, OR, USA). Biomatrix I is from Biomedical Technologies Inc. (Stoughton, MA, USA). Dextran 70 (Macrodex) and Ficoll-Paque are obtained from Pharmacia Biotech AB (Uppsala,

15   Sweden). The basal culture medium consisted of a 1:1 mixture of M-199 and RPMI-1640 supplemented with 20% heat inactivated FBS, penicillin (100 U/ml) and streptomycin (100 µg/ml). The monoclonal antibody IB4 against the common β-chain (CD18) of β_2-integrins is provided by Dr. Claes Lundberg, Pharmacia & Upjohn (Uppsala, Sweden) by courtesy of Dr. Samuel D. Wright, Rockefeller University. F(ab')$_2$ fragment of goat anti-mouse IgG is from

20   Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA).

Polyclonal HBP antibodies are prepared according to the following procedure. New Zealand white rabbits are injected subcutaneously with 50 µg of human heparin-binding protein (HBP) emulsified in Freund's Complete Adjuvant (FCA), followed by three booster injections with 50

25   µg human HBP emulsified in Freund's Incomplete Adjuvant (FIA) every two weeks. Ten days after the last injection, the animals are bled and serum is isolated.

Monoclonal HBP antibodies are prepared according to the following procedure. RBF mice are immunized by injecting subcutaneously 20 µg of purified human HBP in FCA followed by

30   two injections with 20 µg of human HBP in FIA. High responder mice are boosted intravenously with 20 µg of HBP and the spleen is harvested after three days. Spleen cells are fused with the myeloma Fox cell line. Supernatants are screened for anti-HBP antibody production in an HBP-specific ELISA. Positive lines are screened for binding to the intracellular part of a macrophage line by standard FACS methodology. Positive lines are cloned and

retested in both assays. Monoclonal antibodies are purified by Protein A affinity chromatography.

Endothelial cells

5   Human umbilical vein endothelial cells (HUVEC) are isolated from fresh umbilical cords as described by Jaffe et al., 1973, J. Clin. Invest. 52:2745-2756), and bovine aorta endothelial cells (BAEC) are isolated according to the method of Booyse et al., 1975, Thromb. Diath. Haemmorrh. 24:825-839) with a few modifications. Briefly, the lumen of the umbilical vein or bovine aorta is incubated with a 0.25% collagenase solution for 10 min, and detached endo-

10   thelial cells washed out with PBS. Harvested cells are pelleted and resuspended in culture medium, seeded in tissue culture flasks and incubated under standard conditions. Medium is changed every 48 h until cultures have reached confluence (3-5 days). HUVEC or BAEC in the first to fifth passages are used in the experiments. Identification and purity determination of ECs are based on cobblestone morphology and characterization by factor VIII/vWf antigen

15   (Booyse et al., 1975, Thromb. Diath. Haemmorrh. 24:825-839). Staining for factor VIII/vWf factor is homogeneous in the cells.

Seeding onto permeable membranes

Cultured EC are detached by brief (2 min) trypsin-EDTA treatment (0.25% trypsin/0.01 %

20   EDTA) and replated onto either 0.2 μm pore size Anopore inorganic membrane or 3.0 μm pore size polycarbonate filters (Tissue Culture inserts, 10 mm; NUNC, Roskilde, Denmark). In order to promote cellular differentiation and enhance attachment of EC, the filters are treated with 50 μl Biomatrix I (167μg/ml) and left to air-dry. EC are seeded at a density of 2×10^5 cells per filter and incubated in culture medium at 37°C in a humidified atmosphere of 5%

25   CO_2 in air. The ECs are grown to confluent monolayers, as assessed by daily microscopic observation and measurement of the electrical resistance across the monolayer.

Measurement of transendothelial electrical resistance (TEER)

For measurement of electrical resistance across the EC monolayer, the filter insert is trans-

30   ferred to a resistance measurement chamber (ENDOHM-12; World Precision Instruments, Sarasota, FL, USA) modified so as to assure exact positioning of the electrodes in relation to each other and to the filter insert during repeated measurements. The chamber (lower compartment) and the filter insert (upper compartment) are filled with 2 ml and 400 μl culture medium, respectively. All measurements are made at 37°C with the equipment placed in a

cell culture incubator. Direct readings of the electrical resistance are given by an ohmmeter (EVOM; World Precision Instruments, Sarasota, FL, USA) connected to the electrodes of the resistance chamber. The electrical resistance of individual EC monolayers is obtained by subtracting the resistance of the corresponding naked filter coated with Biomatrix as meas-
5   ured prior to seeding of the endothelial cells.

Determination of albumin clearance

Evans blue dye-conjugated albumin (EBA) is used as a marker for macromolecular perme-
ability of the EC monolayers. The dye at a final concentration of 0.67 mg/ml is mixed with
10   culture medium containing 4% BSA. In experiments where albumin permeability is analyzed, the culture medium in the upper compartment is exchanged for medium containing EBA. The EBA concentration in fluid samples of the upper and lower compartment is determined spec-
trophotometrically through measurement of absorbency at 620 nm (Titertek Multiskan MCC; Flow Laboratories, Solna, Sweden). Albumin clearance is calculated according to the rela-
15   tionship: V1 = A2 x V2 x 1/A1, where V1 is the clearance volume (i.e. the theoretical volume of the apical medium cleared from albumin by diffusion to the basolateral compartment), V2 is the basolateral volume, and A1 and A2 is the absorbency of the apical and basolateral medium, respectively. The baseline clearance in absence of any stimulus, averaging 0.08 ± 0.01 µl/min, is subtracted from the clearance volume obtained in response to respective spe-
20   cific stimulus.

PMN isolation and activation

Leukocyte-rich plasma, obtained from human whole blood by dextran sedimentation, is care-
fully layered onto Ficoll-Paque and centrifuged at 400g for 30 min. The PMN-containing pel-
25   let is resuspended and washed twice (400g, 7 min) in ice cold PBS. The PMNs are resus-
pended in culture medium at a final concentration of 2-5 x 10^6 cells/ml. Purity of PMNs is > 98% and viability by Trypan blue exclusion is >95%.

Antibody cross-linking of CD18 molecules on the neutrophil surface is induced as described
30   above. In brief, purified PMNs (5 x 10^6/ml) are incubated with anti-CD18 mAb IB4, 10 µg/ml in HBSS for 30 min. During the incubation period, the cells are subjected to temperature transition from 4° to 37° C in order to increase surface expression of CD11/CD18. The cells are washed twice and cross-linking of CD18 is accomplished through addition of F(ab')₂ fragments of goat anti-mouse IgG (diluted 1:20). The PMNs are sedimented by centrifugation

at 300g for 15 min at room temp, and the cell-free supernatant is collected. In separate experiments, antibody cross-linking of CD18 is induced after PMNs preincubated with mAb IB4 are added to EC monolayers (see below).

Experimental procedures

Before transferring the filter inserts to the resistance measurement chamber (kept at 37° C), the medium in the insert and in the chamber is replaced with fresh culture medium (37°C) supplemented with 10 mM Hepes. HBP (25-100 µg/ml) or PMN secretion product is added either to the upper or the lower compartment and change in transendothelial electrical resistance is measured every minute until a plateau phase is reached, and thereafter at 5-min intervals. In separate experiments, PMNs (2×10^6) preincubated with mAb IB4 (10 µg/ml) are added to the upper compartment (PMN:EC ratio = 10:1) and allowed to sediment for 10 min. Activation of the PMNs is induced through antibody crosslinking of leukocytic CD18 by addition of goat anti-mouse IgG F(ab')$_2$ diluted 1:20. In experiments where albumin permeability across the EC monolayer is studied, the medium in the insert contained EBA. During the experiment, the filter insert is moved at 5-10 min intervals to new wells containing fresh medium. All incubations are made at 37°C. After the experiment, the medium in the lower wells is aspirated and analyzed for EBA content.

Determination of EC intracellular Ca^{2+} and F-actin distribution

The Ca^{2+} sensitive fluorescent probe fluo-3/AM is used for determination of changes in EC intracellular free Ca^{2+}. Confluent HUVEC or BAEC monolayers cultured on Biomatrix I coated filters are incubated with a Fluo-3/AM solution (3µM in HBSS containing 2% FCS and 10 µM HEPES), added to both the apical and basolateral surface, for 30 min at 37° C. The monolayers are washed three times and incubated with fresh HBSS in the dark for 20 min at room temperature before use to allow complete hydrolysis of the dye ester to its calcium sensitive free acid form. EC are stimulated with HBP added to the apical surface. Changes in EC $[Ca^{2+}]_i$ in response to HBP stimulation are measured through continuous registration of fluorescence intensity with a laser-scanning confocal imaging system (Insight Plus; Meridian Instruments Inc., Okemon, Michigan).

For analysis of F-actin distribution in EC in response to stimulation with HBP, confluent EC monolayers grown on Biomatrix coated coverslips are incubated with HBP or vehicle alone at 37° C for 15 min. The monolayers are washed twice in culture medium and fixed in 3.7%

formaldehyde in PBS for 10 min at room temperature. EC are permeabilized with 0.2% Triton X-100 in PBS (1 min at 4^0 C), washed twice and stained for actin filaments with FITC-conjugated phalloidin for 20 min at 37^0 C. The endothelial cells are again washed three times in PBS and viewed in a laser scanning confocal microscope.

Western blot analysis
A sample of purified HBP or PMN-secretion is added to SDS sample buffer, and boiled for 5 min. Electrophoresis is performed by applying the samples to SDS polyacrylamide gradient gels (3-10 %). Proteins are electrophoretically transferred onto a nitrocellulose membrane (Bio-Rad Laboratories, Richmond, CA) for immunoblot analysis. Additional binding sites are blocked by incubating the nitrocellulose membrane for 2 hr. in milk. HBP is detected using western blot analysis.

RESULTS

Activated PMN release HBP
PMNs in suspension are activated through antibody cross-linking of CD18. The PMNs are centrifuged and the supernatant is collected and run on SDS-PAGE. Western blot analysis using antibodies directed against HBP recognized a prominent band of 27 kD in the PMN secretion. No specific bands are found with control rabbit IgG.

HBP increases permeability of EC monolayers
Addition of HBP (75 µg/ml) to the upper compartment elicited a marked decrease in transendothelial resistance, which is manifest within one min, reached its maximum (30% ± 3% of control) after 15 min, and so remained throughout the observation period (Figure 2). Challenge with HBP also caused an increase in EC macromolecular permeability, as reflected in a progressive rise in albumin clearance yielding a net clearance volume of 20.4 ± 3.4 µl over a 60 min period (Figure 2). The EC responses to HBP stimulation are clearly dose-dependent as illustrated by less pronounced decreases in TEER at steady state with HBP 25 and 50 µg/ml (Figure 2). However, the rate of change by which the responses occur is the same for all doses of HBP.

Stimulation of EC with HBP from the basolateral side of the monolayer (HBP added to the lower compartment) elicited similar changes in EC permeability as for apical stimulation, however at somewhat higher concentrations.

5   *Neutralization of HBP attenuates PMN-induced increase in EC permeability*
Purified IgG of a rabbit antiserum against HBP is used to neutralize the activity of the protein. Addition of HBP antibodies (50 µg/ml) to the EC monolayer prior to stimulation with HBP (75 µg/ml) markedly attenuated the evoked changes in TEER and macromolecular permeability. The change in TEER occurred with some latency and reached to 75% ± 5% of control (Fig-
10   ure 3), signifying an inhibition of the HBP-induced response by approximately 65 %. Simi-larly, the albumin efflux is inhibited by approximately 60 % to a net clearance volume of 8.64 ± 1.74 µl over 60 min (Figure 3). Treatment with non-specific rabbit IgG (50 µg/ml) is without effect on HBP-induced decrease in TEER, reaching 31% ± 5% of control in 15 min.

15   A determination is made whether antibodies against HBP could exert inhibitory actions also on changes in EC barrier function triggered by activated PMNs. PMNs in suspension are ac-tivated through antibody cross-linking of leukocytic CD18 and centrifuged. The supernatant containing PMN secretion products (see Materials and Methods) is transferred to the EC monolayer. In the presence of antibodies against HBP, the decrease in TEER and increase
20   in macromolecular permeability are markedly suppressed as compared with the responses to stimulation with PMN secretion in absence of anti-HBP (Figure 4). Almost identical effects are obtained when antibody cross-linking of CD18 is induced after PMNs have been added to the EC monolayer and hence, the PMN secretion product directly reached the EC mono-layer.

25

HBP stimulation increases EC intracellular free Ca^{2+}
Laser scanning confocal microscopy is employed to determine if EC responded to HBP stimulation by an increase in cytosolic Ca^{2+}. Addition of HBP (75 µg/ml) to confluent HUVEC or BAEC monolayers, loaded with the Ca^{2+} sensitive fluorophore fluo-3/AM, provoked a rapid
30   increase in fluorescence intensity, which peaked within 1 min and then gradually declined toward baseline levels. This intracellular Ca^{2+} mobilization clearly indicates an active re-sponse of EC to HBP stimulation.

HBP stimulation induces reorganization of EC microfilaments

Confluent EC monolayers incubated with HBP (75 µg/ml) or culture medium alone for 15 min
are stained for actin filaments with FITC-conjugated phalloidin and viewed in a laser scan-
ning confocal microscope. In comparison with untreated EC, exhibiting low density of F-actin
5   stress fibers and distinct dense peripheral bands along the cell margin, there are substantial
changes in F-actin content and distribution in the HBP stimulated EC.   An increased density
of F-actin stress fibers spanning the cell and disruption of peripheral bands indicated reor-
ganization of the EC cytoskeleton as a consequence of HBP stimulation.

10   ### HBP increases vascular permeability in vivo

Topical application of HBP (75 µg/ml) to the hamster cheek pouch microvasculature results
within 2 min in visible leaky spots of the fluorescent tracer of both postcapillary and larger
venules. The maximum leakage response is seen at 7-10 min after challenge. The leakage
then subsided slowly and disappeared completely within 30 min.

15

Inhibition of Bradykinin- and HBP-Induced Increase in EC Permeability with Mono-clonal Antibody MBK3 to Bradykinin

Bradykinin (100 nM; Figure 5a) or HBP (75 µg/ml; Figure 5b) is administered at time zero to
the luminal side of EC monolayers pre-incubated with a monoclonal antibody to bradykinin,
20   MBK3 (40 µg/ml) (Haasemann et al., 1991, J. Immunol. 147:3882-3892). MBK3 totally pre-
vented the increase in EC permeability induced by either bradykinin or HBP. The specificity
of the mAb effect is documented by the lack of inhibition of histamine-induced permeability
change (histamine administered at 15 min after stimulation with bradykinin or HBP), N=6.

25   ### Inhibition of HBP-induced increase in EC permeability with monoclonal antibody PKH4 to plasma kallikrein

HBP (75 µg/ml; filled symbols) or bradykinin (100 nM; open symbols) is administered at time
zero to the luminal side of EC monolayers pre-incubated with mAb PKH4 (40 µg/ml). The
results are shown in Figure 6. PKH4 prevented the increase in EC permeability induced by
30   HBP, but not that induced by bradykinin. PKH4-treatment did not affect the permeability
change induced by histamine (histamine administered at 15 min after stimulation with HBP),
N=6.

Inhibition of HBP-induced increase in EC permeability by peptide HKH20-treatment

EC monolayers are incubated with HKH20 (3 µg/ml) for 30 min, which treatment displaces
surface-bound H-kininogen. After wash, HBP (75 µg/ml) is administered at time zero to the
luminal side of ECs followed by bradykinin (100 nM) at 15 min. HK is anchored to the EC sur-
5   face by domain 5 and 6, with domain 5 being the most important. The minimum amino acid
sequence required for human HK binding in domain 5 to EC is represented by a 20 amino
acid peptide (HKH 20) with the following sequence: HKHGHGHHKKNKGKKNGKH (SEQ ID
NO:7) (human H-kininogen 479-498). This peptide inhibits the attachment of HK to EC
(Hasan et al., 1995, J. Biol. Chem. 270:19256-19261 and Herwald et al., 1996, J. Biol.
10   Chem. 271:13040-13047). The results are shown in Figure 7. HKH20-treatment of EC pre-
vented HBP-induced increase in EC permeability, but not the permeability change induced
by subsequent administration of bradykinin, N=6.

Inhibition of HBP-induced increase in EC permeability by peptide SDD31-treatment

15   EC monolayers are incubated with SDD31 (3 µg/ml) for 30 min. SDD31, also referred to as
HK31, is a peptide derived from human H-kininogen HK) domain 6, which blocks the binding
of HK to plasma kallikrein and factor XII (Tait et al, 1986, J. Biol. Chem. 261:15396-15401
and Vogel et al., 1990, J. Biol. Chem. 265:12494-12502). It has the sequence SDDDWIP-
DIQTDPNGLSFNPISDFPDTTSPK(SEQ ID NO:8) (human H-kininogen 565-595). After wash,
20   HBP (75 µg/ml) is administered at time zero to the luminal side of EC followed by bradykinin
(100 nM) at 15 min. The results are shown in Figure 8. SDD31-treatment of EC largely pre-
vented HBP-induced increase in EC permeability. The EC response to direct stimulation with
bradykinin persisted after SDD31-treatment, N=6.

25   *Inhibition of HBP-induced increase in EC permeability by aprotinin-treatment*

HBP (75 µg/ml) is administered at time zero to the luminal side of EC monolayers pre-
incubated with aprotinin (100 µg/ml; 30 min). The results are shown in Figure 9. Aprotinin-
treatment largely prevented HBP-induced increase in EC permeability, but not the permeabil-
ity change induced by subsequent administration of bradykinin (100 nM), N=6.

30

An HBP mutant (G175N) is unable to bind aprotinin due to the mutation but the three dimen-
sional structure and the special surface charge distribution is unaltered. When tested in the
leakage model, this mutant induces bradykinin release and increase in EC permeability.
However, addition of aprotinin has no inhibitory effect. A similar result is obtained with por-

cine HBP which is very homologous to human HBP but is unable to bind aprotinin due to the presence of an R in the bottom of the degenerated catalytic cleft.

Example 3: Two Mutants of Heparin-Binding Protein

5

Two mutants of human HBP, [R23S,F25E]HBP and [G175Q]HBP, have been produced to investigate structure-function relationships of residues in the putative lipid A/LPS binding site and BPTI (bovine pancreatic trypsin inhibitor) binding site. The G175Q mutation does not alter the structure of the protein, but the ability to bind BPTI has been eliminated, and the

10   mutant mediates only a very limited stimulation of the lipopolysaccharide (LPS) induced cy-tokine-release from human monocytes. The lipid A/LPS binding property is comparable to that of native HBP. The R23S,F25E mutations do not affect the binding of lipid A/LPS and BPTI or the lipopolysaccharide (LPS) induced cytokine-release from human monocytes.

15   **MATERIALS AND METHODS**

Construction of mutant proteins

Pwo DNA polymerase is from Boehringer Mannheim, *Bam*H1 and T4 DNA ligase are from New England Biolabs, *spodoptera frugiperda* (*sf*9) cells and pVL1393 transferplasmid are

20   from Invitrogen, TC100 and SF900-II are produced at Novo Nordisk A/S, Foetal calf serum (FCS) is from GibcoBRL. All other chemicals are of the finest grade available.

[R23S,F25E]HBP mutant

The [R23S,F25E]HBP mutant is constructed using the PCR overlap-extension technique

25   (Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K., and Pease, L. R. (1989) *Gene (Amst.)* 77: 51-59). Two partially overlapping mutated fragments are prepared by PCR using oligonucleo-tides 1+2 and 3+6 and the resulting fragments are joined using oligonucleotide 1+6 (Table 1). Native human HBP cDNA is used as template for the PCR reaction. The resulting cDNA en-coded the 19 amino acid pro-peptide, the 222 amino acid mature part and a three amino ac-

30   ids C-terminal extension. *Bam*H1 sites are incorporated on each side of the mutated cDNA for cloning purposes. The mutated fragment is ligated into the pVL1393 transfer plasmid (In-vitrogen). DNA for transfection is prepared using the Qiagen midiprep kits. Following purifica-tion, the insert is sequenced.

Table 1:   Oligonucleotides used for construction of the

                [R23S,F25E]HBP and [G175Q]HBP mutants

No.	Sequence
1.	5'-CCGGGGATCCGATGACCCGGCTGACAGTCCTGG-3' (SEQ ID NO:9)
2.	5'-GGCACCCCCGCACTCGTGGCTGCCTTGATTCTG-3' (SEQ ID NO:10)
3.	5'-CAGAATCAAGGCAGCCACGAGTGCGGGGGTGCC-3' (SEQ ID NO:11)
4.	5'-GAGGGGGGTGCCCTGGTCCCCATTGCA-3' (SEQ ID NO:12)
5.	5'-TGCAATGGGGACCAGGGCACCCCCCTC-3' (SEQ ID NO:13)
6.	5'-CCGGGGATCCAACTAGGCTGGCCCCGGTCCCGG-3' (SEQ ID NO:14)

[G175Q]HBP mutant.

This mutant is in general constructed as described above. Two partially overlapping mutated
fragments are prepared by PCR using oligonucleotides 1+4 and 5+6 and the resulting frag-
ments are joined using oligonucleotides 1+6 (Table 1).

Expression and purification

_sf_9 insect cells are maintained in TC100 medium supplied with 10% FCS. Prior to infection,
the medium is changed to serum-free SF900-II. Expression of both mutants in serum-free
medium and purification of the mutants from the culture supernatant is performed as previ-
ously described for native human HBP (Rasmussen, P. B., Bjørn, S., Hastrup, S., Nielsen, P.
F., Norris, K., Thim, L., Wiberg, F. C., and Flodgaard, H. (1996) _FEBS Lett. 390:_ 109-112).

Assay of LPS induced IL-6 release from monocytes

Human monocytes are isolated and treated as described (Rasmussen, P. B., Bjørn, S., Has-
trup, S., Nielsen, P. F., Norris, K., Thim, L., Wiberg, F. C., and Flodgaard, H. (1996) _FEBS
Lett. 390:_ 109-112), with the exception that 1% non-essential amino acids is excluded from
the medium.

Assay of ^{3}H-LPS binding to HBP

The affinity for lipopolysaccharide (^{3}H-LPS) is measured by scintillation proximity assay tech-
nology (SPA) as described (Linde, V., Bjørn, S., Kastrup, J. S., and Flodgaard, H., 2000, Bio-
5   techniques 28:218-222). All samples are repeated in triplicates.

Assay of ^{125}I-BPTI binding to HBP

The affinity for BPTI is assessed using a SPA assay essentially as described for the LPS as-
say (Linde, V., Bjørn, S., Kastrup, J. S., and Flodgaard, H. , 2000, Biotechniques 28:218-
10   222) with a few modifications. ^{3}H-LPS is exchanged for ^{125}I-BPTI (prepared by Novo Nordisk
A/S) and the buffer is changed to PBS (20 mM phosphate pH 7.4 and 150 mM NaCl) includ-
ing 0.05 % BSA and 0.05 % Triton X-100. The relative affinities of HBP and the mutants are
determined by performing competition studies with fixed, increasing concentrations of unla-
beled BPTI.

15

RESULTS

Biological characterisation

The biological activity of the two mutants are compared to native HBP with respect to their
ability to enhance, in a dose-dependent manner, the LPS-induced II-6 release from human
20   monocytes *in vitro*. The LPS affinity of the two mutants and of HBP are measured in a scintil-
lation proximity assay (SPA) (Linde, V., Bjørn, S., Kastrup, J. S., and Flodgaard, H., 2000,
Biotechniques 28:218-222). Both mutants bind ^{3}H-LPS in a saturable manner with similar
apparent K_d (approximately 0.7   g/mL) as observed for native HBP (Figure 11a). Further-
more, the affinity for BPTI is measured for both mutants and for native HBP in a similar scin-
25   tillation proximity assay, where ^{125}I-BPTI is used as ligand for HBP. The [R23S,F25E]HBP
mutant as well as native HBP binds BPTI whereas no binding is observed for the
[G175Q]HBP mutant (Figure 11b). Competition studies performed by addition of fixed, in-
creasing concentrations of unlabeled BPTI showed that ^{125}I-BPTI binds to HBP and
[R23S,F25E]HBP with similar apparent IC_{50} values of approximately 7 nM. As observed in
30   Figure 11c, [R23S,F25E]HBP apparently has a larger binding capacity as compared to HBP.
This difference is probably due to an ability of [R23S,F25E]HBP to bind different forms of the
iodinated BPTI used. However, the larger binding capacity does not influence the affinity of
^{125}I-BPTI. In addition, the ability of ^{3}H-LPS to displace ^{125}I-BPTI bound to native HBP, and

vice versa, has been investigated by competition experiments: competition for ^{125}I-BPTI bind-ing by adding up to 5 µg/mL unlabeled LPS and competition for ^{3}H-LPS binding by adding up to 50 mM unlabeled BPTI. In both situations no inhibitory effects are observed, indicating that the LPS and BPTI binding sites are located at different parts of the HBP molecule.

The biological *in vitro* activity assay data show that [G175Q]HBP, contrary to [R23S,F25E]HBP and native HBP, mediates only a very limited stimulation of the lipopoly-saccharide (LPS) induced cytokine-release from human monocytes (Figure 10).

The invention described and claimed herein is not to be limited in scope by the specific em-bodiments herein disclosed, since these embodiments are intended as illustrations of several aspects of the invention. Any equivalent embodiments are intended to be within the scope of this invention. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing descrip-tion. Such modifications are also intended to fall within the scope of the appended claims.

Various references are cited herein, the disclosures of which are incorporated by reference in their entireties.

CLAIMS

1.   A method for preventing or treating a disorder resulting from release of bradykinin in a mammal, wherein said mammal produces HBP that binds to an HBP antagonist, said method comprising administering to said mammal in need thereof, a mammalian HBP antagonist in an amount effective to decrease release of bradykinin in said mammal.

2.   The method according to claim 1, in which the HBP antagonist is present in an amount of from about 10 mg to about 1 g per unit dosage form.

3.   The method according to claim 1, in which the HBP antagonist is present in an amount of about 0.1-100 mg/kg body weight.

4.   The method according to claim 1, in which the HBP antagonist is present in an amount of about 0.5-50 mg/kg body weight.

5.   The method according to claim 1, in which the HBP antagonist is present in an amount of about 1-25 mg/kg body weight.

6.   The method according to claim 1, in which the disorder is selected from the group consisting of systemic inflammatory response syndrome, ischemia reperfusion, anaphylasis and allograft rejection.

7.   The method according to claim 1, in which the disorder is adult respiratory distress syndrome.

8.   Use of a mammalian HBP antagonist for the preparation of a medicament for preventing or treating a disorder resulting from release of bradykinin in a mammal wherein said mammal produces HBP that binds to an HBP antagonist.

9.   The use according to claim 8, in which the HBP antagonist is present in an amount of from about 10 mg to about 1 g per unit dosage form.

10.   The use according to claim 8, in which the HBP antagonist is present in an amount of about 0.1-100 mg/kg body weight.

11.   The use according to claim 8, in which the HBP antagonist is present in an amount of
5   about 0.5-50 mg/kg body weight.

12.   The use according to claim 8, in which the HBP antagonist is present in an amount of about 1-25 mg/kg body weight.

10   13.   The use according to claim 8, in which the disorder is selected from the group consisting of systemic inflammatory response syndrome, ischemia reperfusion, anaphylaxis and allograft rejection.

14.   The use according to claim 8, in which the disorder is adult respiratory distress syn-
15   drome.

15.   A method for preventing or treating a disorder resulting from release of bradykinin in a mammal, wherein said mammal produces HBP that binds to aprotinin or analog thereof, said method comprising administering to said mammal in need thereof, aprotinin or an analog
20   thereof, in an amount effective to decrease release of bradykinin in said mammal.

16.   The method according to claim 15, wherein said aprotinin analog is an aprotinin vari- ant containing at least one mutation in the 15-19 position.

25   17.   The method according to claim 15, in which the disorder is selected from the group consisting of systemic inflammatory response syndrome, ischemia reperfusion, anaphylaxis and allograft rejection.

18.   The method according to claim 15, in which the disorder is adult respiratory distress
30   syndrome.

19.   Use of aprotinin or an analog thereof for the preparation of a medicament for decreasing release of bradykinin in a mammal wherein said mammal produces HBP that binds to aprotinin or an analog thereof.

20.   The use according to claim 19 wherein said aprotinin analog is an aprotinin variant containing at least one mutation in the 15-19 position.

5   21.   Use of aprotinin or an analog thereof, for the preparation of a medicament useful for the treatment of systemic inflammatory response syndrome, ischemia reperfusion, anaphylaxis and allograft rejection in a patient having a HBP that binds to aprotinin or an analog thereof.

10   22.   Use of aprotinin or analog thereof, for the preparation of a medicament useful for the treatment of adult respiratory distress syndrome in a patient having a HBP that binds to aprotinin or an analog thereof.

23.   A method for preventing or treating a disorder resulting from release of bradykinin in a
15   mammal, wherein said mammal produces HBP that binds to a monoclonal antibody that binds at least one epitope of HBP, wherein said epitope binds to prekallikrein-H-kininogen complex and activates release of bradykinin, said method comprising administering to said mammal in need thereof, a monoclonal antibody that binds at least one epitope of HBP, wherein said epitope binds to prekallikrein-H-kininogen complex and activates release of bra-
20   dykinin in an amount effective to decrease release of bradykinin in said mammal.

24.   The method according to claim 23, wherein said mammal is a human patient and said HBP is human HBP.

25   25.   The method according to claim 23, wherein said monoclonal antibody is a human monoclonal antibody.

26.   Use of a monoclonal antibody that binds at least one epitope of HBP wherein said epitope binds to prekallikrein-H-kininogen complex and activates bradykinin for the prepara-
30   tion of a medicament for preventing or treating a disorder resulting from release of bradykinin in a mammal wherein said mammal produces HBP that binds to said monoclonal antibody that binds at least one epitope of HBP wherein said epitope binds to prekallikrein-H-kininogen complex and activates bradykinin.

27.   The use according to claim 26, wherein said mammal is a human patient and said HBP is human HBP.

28.   The use according to claim 26, wherein said monoclonal antibody is a human mono-
5   clonal antibody.

29.   The use according to claim 26 for the preparation of a medicament useful for the treatment of systemic inflammatory response syndrome, ischemia reperfusion, anaphylaxis and allograft rejection.
10

30.   The use according to claim 26 for the preparation of a medicament useful for the treatment of adult respiratory distress syndrome.

31.   A method of identifying an antagonist of HBP, said method comprising (a) culturing en-
15   dothelial cells in the presence of HBP and in the presence and absence of a substance sus-
pected of being said antagonist and (b) detecting any effect of said substance on permeabil-
ity of endothelial cells, wherein decreased permeability of said endothelial cells as compared to permeability of said cells when incubated in the presence of HBP without said substance indicates that said substance is an antagonist.
20

32.   The method according to claim 31, wherein in step (a) endothelial cells are first cul-
tured in the presence of HBP and permeability of said cells are measured and then said cells are subsequently cultured in the presence of said substance.

25   33.   The method according to claim 31, wherein in step (a), endothelial cells are first cul-
tured in the presence of said substance and then said cells are subsequently cultured in the presence of said HBP.

34.   The method according to claim 31, wherein one sample of endothelial cells are cultured
30   in the presence of HBP and a second sample is cultured in the presence of HBP and said substance.

35.   The method according to claim 31, in which the mammalian HBP is a human HBP.

36.   A method for identifying a HBP antagonist comprising (a) incubating HBP with a first substance that interacts with HBP and a second substance suspected of being a HBP antagonist and (b) detecting any effect of said second substance suspected of being an antagonist on interaction of HBP with said first substance, wherein decreased interaction be-

5   tween said HBP with said first substance is an indication that said second substance is an HBP antagonist.

37.   A method of identifying a monoclonal antibody that binds to at least one epitope on native HBP, wherein said epitope binds to prekallikrein-H-kininogen complex and activates

10   release of bradykinin, said method comprising (a) culturing endothelial cells in the presence of HBP and in the presence and absence of a substance suspected of being said antagonist and (b) detecting any effect of said substance on permeability of endothelial cells, wherein decreased permeability of said endothelial cells as compared to permeability of said cells when incubated in the presence of HBP without said substance indicates that said mono-

15   clonal antibody binds to at least one epitope on said HBP.

38.   The method according to claim 37, wherein said native HBP is a human HBP.

39.   A method of identifying a monoclonal antibody that binds to at least one epitope on

20   native HBP, wherein said epitope binds to prekallikrein-H-kininogen complex and activates release of bradykinin, said method comprising (a) incubating a prekallikrein-H-kininogen complex in the presence of HBP and in the presence and absence of a monoclonal antibody suspected of binding to at least one epitope on said HBP and (b) detecting any effect of said antibody on release of bradykinin, decreased bradykinin release indicating that said antibody

25   binds to at least one epitope on said HBP.

40.   The method according to claim 39, wherein the prekallikrein-H-kininogen complex is attached to a solid support.

30   41.   The method according to claim 39, which further comprises incubating the mixture of step (a) in the presence of endothelial cells.

42.   The method according to claim 39, which further comprises incubating the mixture of step (a) in the presence of platelets.

43.   The method of claim 39, wherein the native HBP is native human HBP.

44.   The method according to claim 39, wherein the release of bradykinin is detected by an immunoassay.

45.   A method for determining if a mammal produces HBP that binds to an HBP antagonist comprising (a) isolating HBP or cells or tissue producing HBP from a patient; (b) incubating said HBP or cells or tissue producing HBP with a substance, tissue, cells or component thereof which interacts with HBP and said HBP antagonist and (c) detecting an effect of said HBP antagonist on the interaction of HBP with said substance, tissue, cells or components thereof, decreased interaction indicating that said HBP binds to said HBP antagonist.

46.   The method according to claim 45, wherein said HBP is first incubated with said antagonist, then subsequently incubated with a substance, tissue, cells or component thereof which interacts with HBP.

47.   The method according to claim 45, wherein said HBP is first incubated with a substance, tissue, cells or component thereof which interacts with HBP and then with said antagonist.

48.   A test kit comprising (a) HBP antagonist; (b) HBP or cell producing HBP and (c) a substance, tissue, cells or component thereof which interact with HBP.

49.   The test kit according to claim 48, wherein the HBP is labeled.

50.   The test kit according to claim 48, wherein the test kit comprises a substance which interacts with HBP.

51.   The test kit according to claim 50, wherein the substance is H-kininogen.

52.   The test kit according to claim 50, wherein the substance is labeled.

53.   The test kit according to claim 50, which further comprises beads.

54.   A method for determining if a mammal produces HBP that binds to a monoclonal an-
tibody that binds to at least one epitope on native HBP, wherein said epitope binds to prekal-
likrein-H-kininogen complex and activates release of bradykinin, comprising (a) isolating HBP
5   or cells or tissue producing HBP from said mammal; (b) culturing said HBP or cells or tissue
producing HBP with endothelial cells in the presence or absence of said antibody and (c) de-
tecting any effect of said antibody on permeability of endothelial cells, decreased permeabil-
ity indicating that said HBP binds to said antibody.

10   55.   The method according to claim 54, in which the mammal is a human patient.

56.   The method according to claim 54, wherein the native HBP is native human HBP.

57.   A method for determining if a mammal produces HBP that binds to a monoclonal an-
15   tibody that binds to at least one epitope on native HBP, wherein said epitope binds to prekal-
likrein-H-kininogen complex and activates release of bradykinin, comprising (a) isolating HBP
or cells or tissue producing HBP from said mammal; (b) incubating said HBP or cells or tis-
sue producing HBP with prekallikrein-kininogen complex and (c) detecting any effect of said
HBP on the release of bradykinin from the prekallikrein-H-kininogen complex, decreased re-
20   lease of bradykinin indicating that said HBP binds to said antibody.

58.   A test kit comprising (a) a monoclonal antibody that binds to an epitope of HBP (b)
native human HBP and (c) a prekallikrein-H-kininogen complex attached to a solid support.

Effect of PMNL on endothelial barrier function
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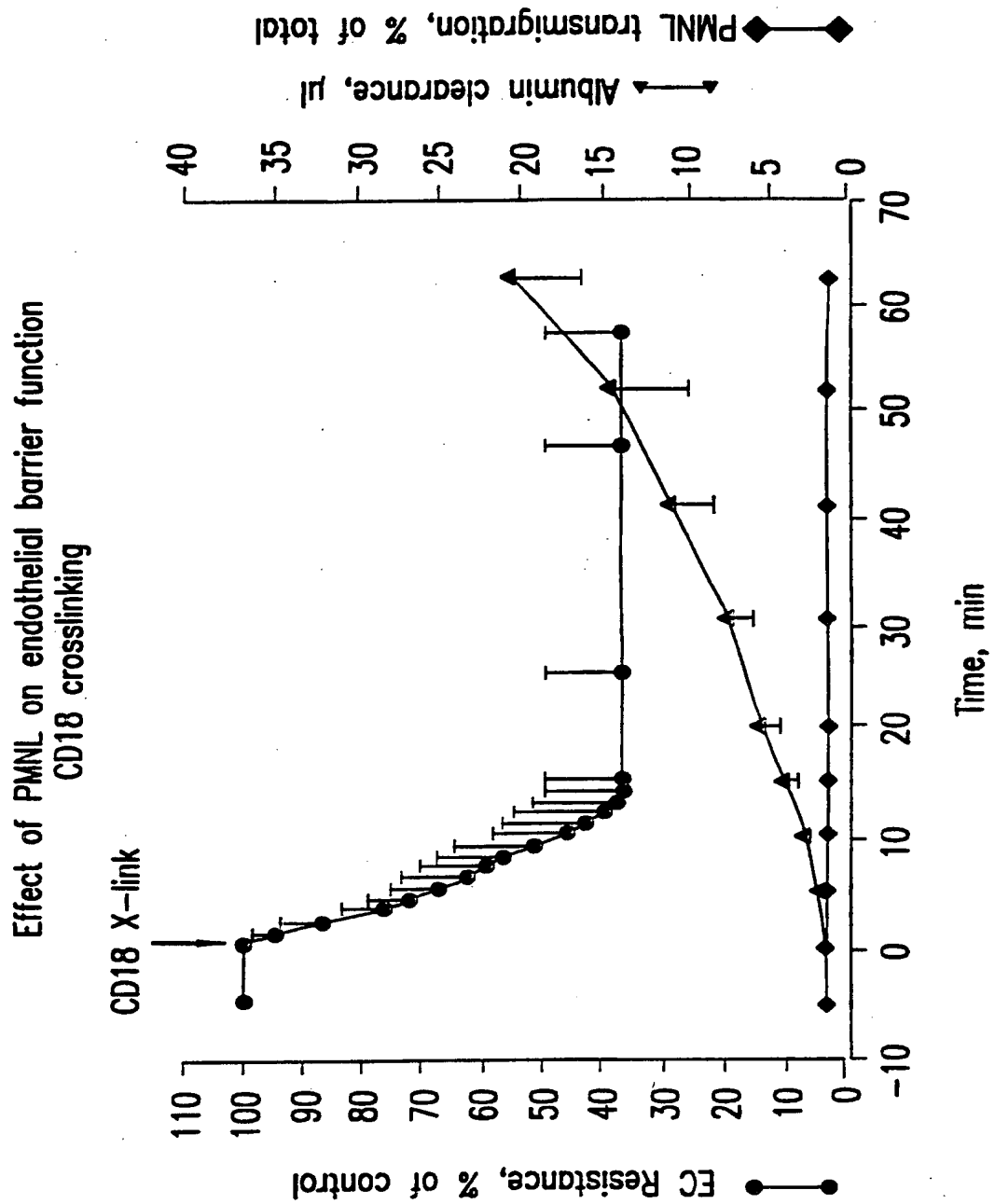
FIG.1

Effect of HBP on endothelial barrier functon
Apical stimulation

FIG.2

Effect of HBP on endothelial barrier function
Anti-HBP treatment
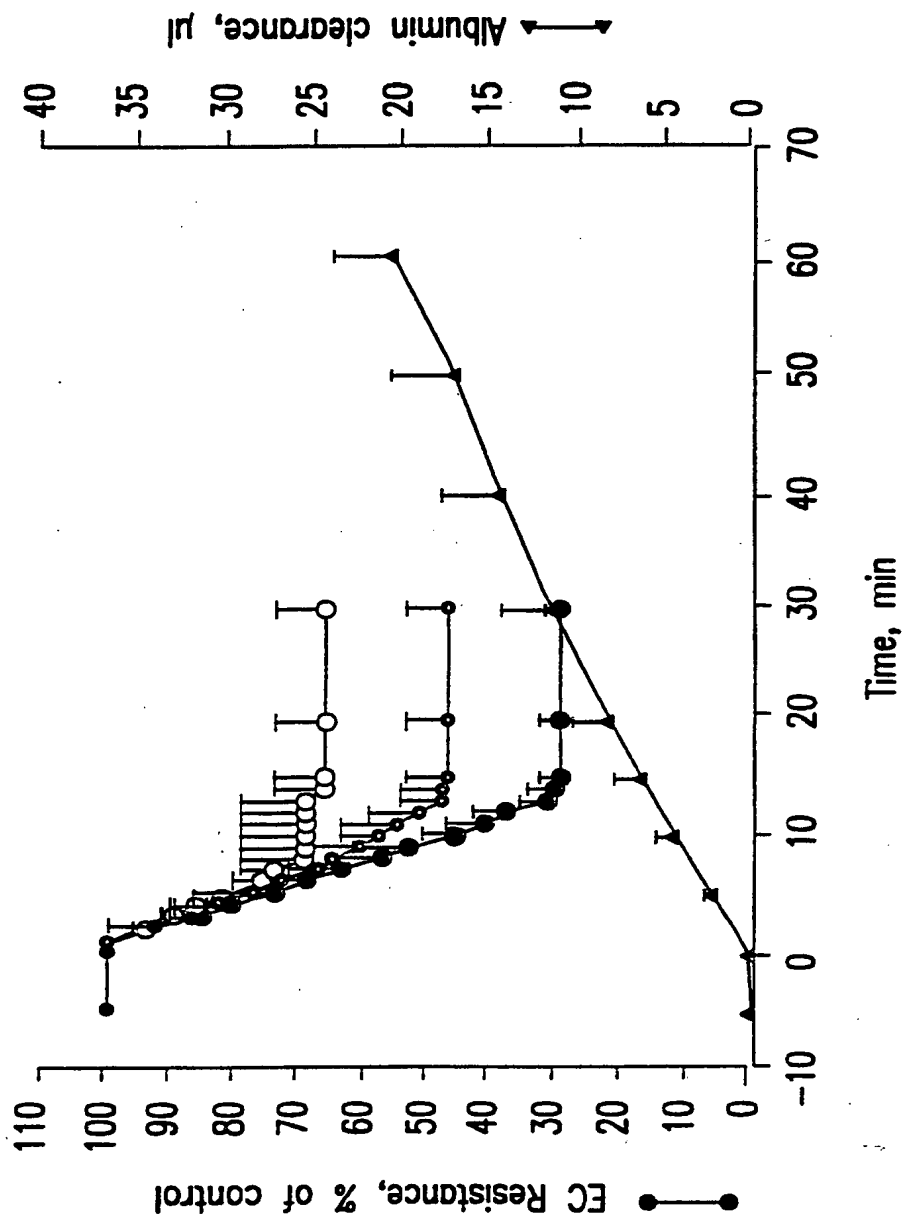
FIG.3

Effect of PMN secretion on endothelial barrier function
Anti-HBP treatment

FIG.4

Bradykinin induced effect on endothelial barrier function
MBK3–treatment

FIG.5a

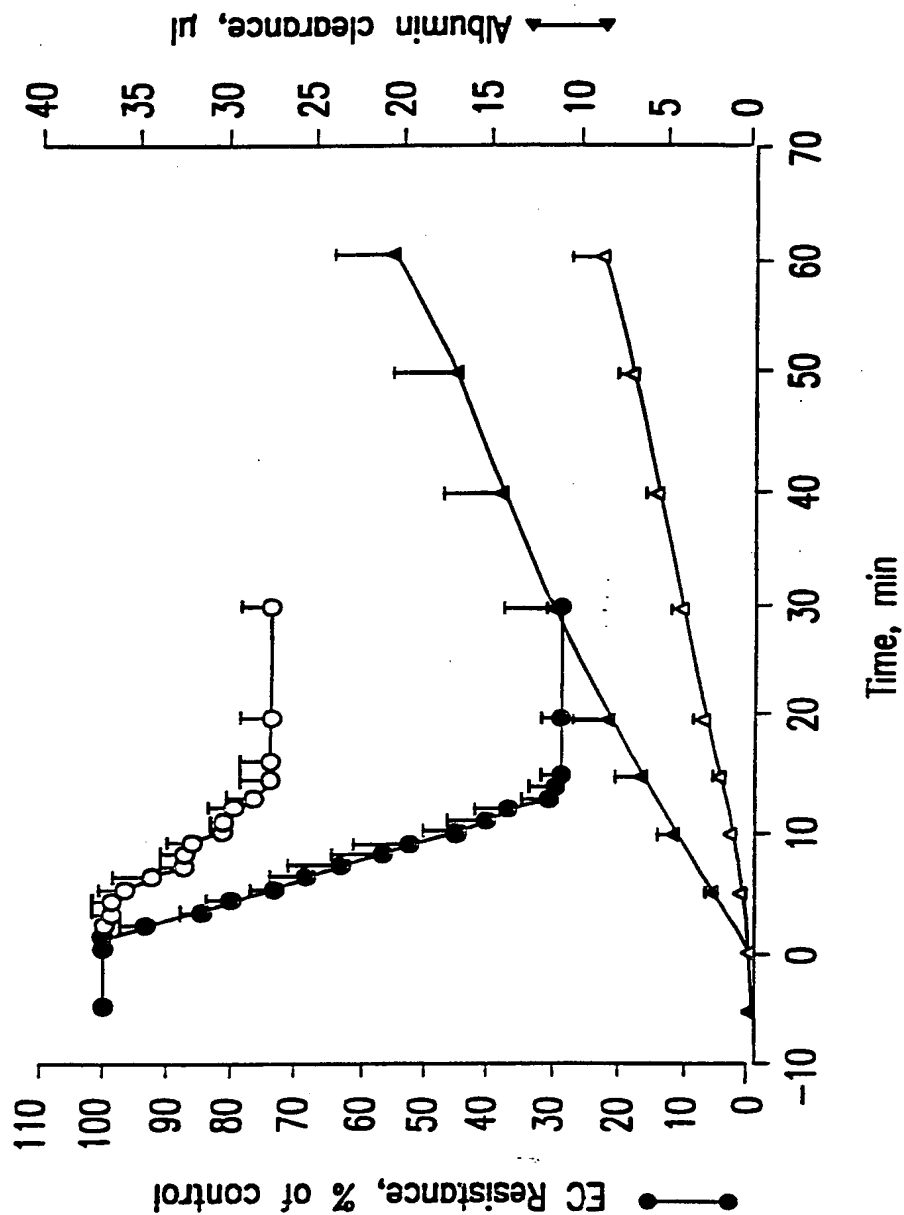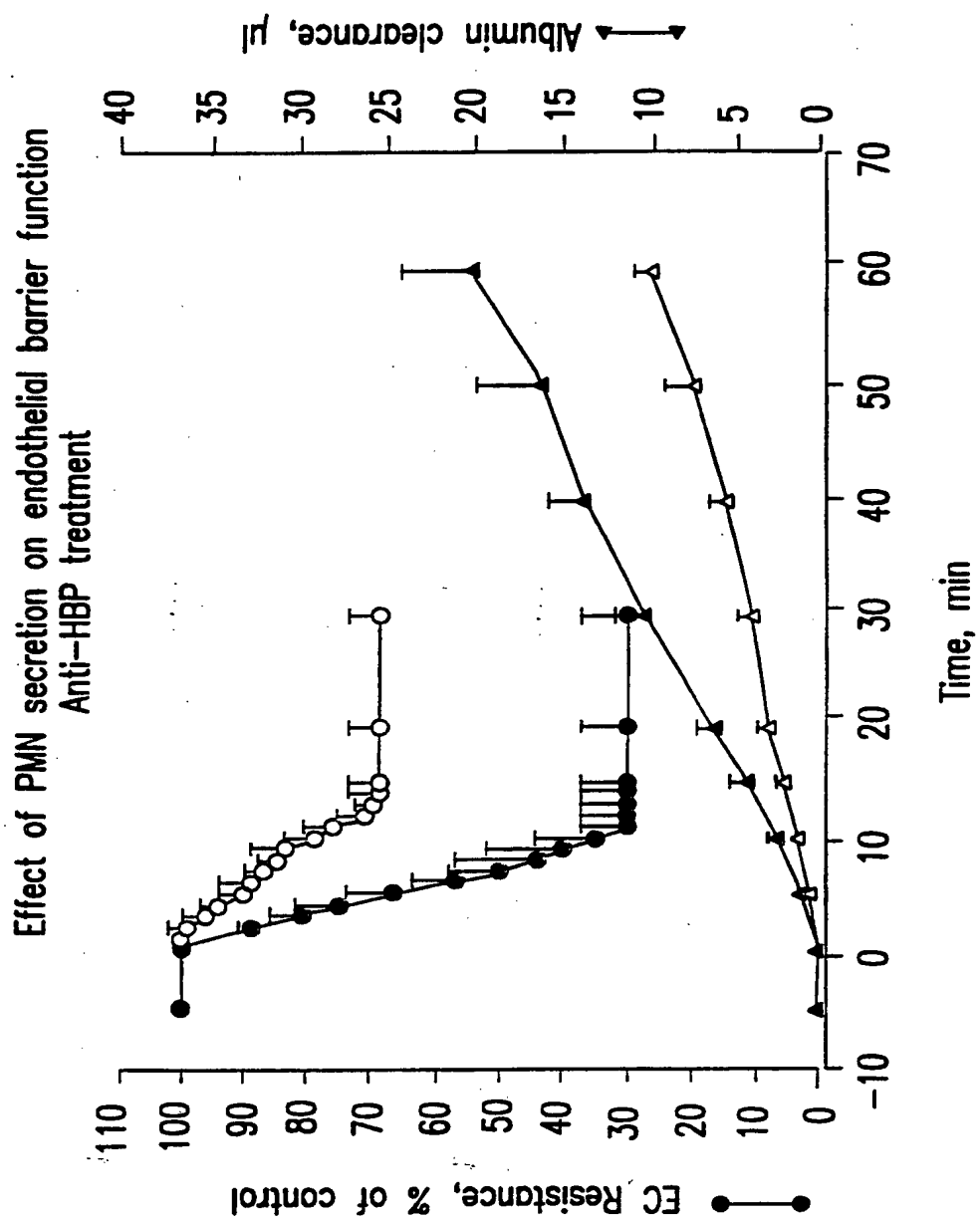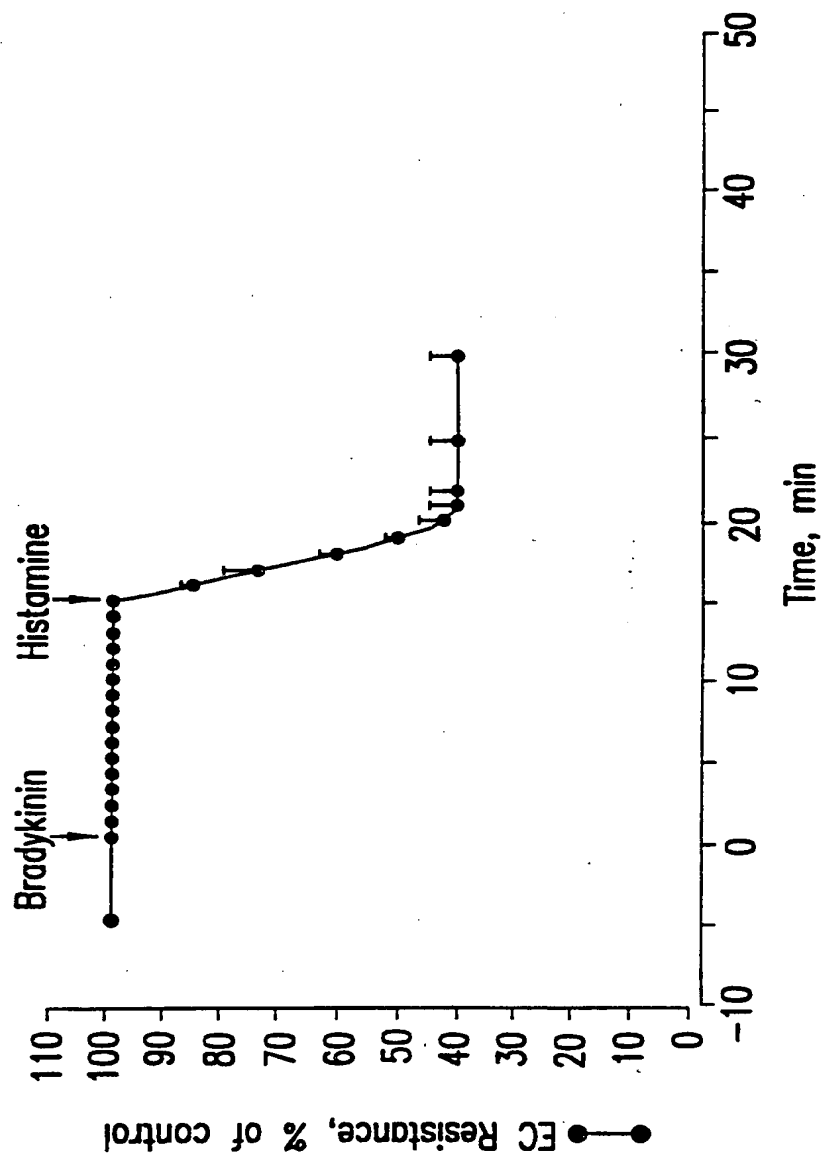HBP induced effect on endothelial barrier function
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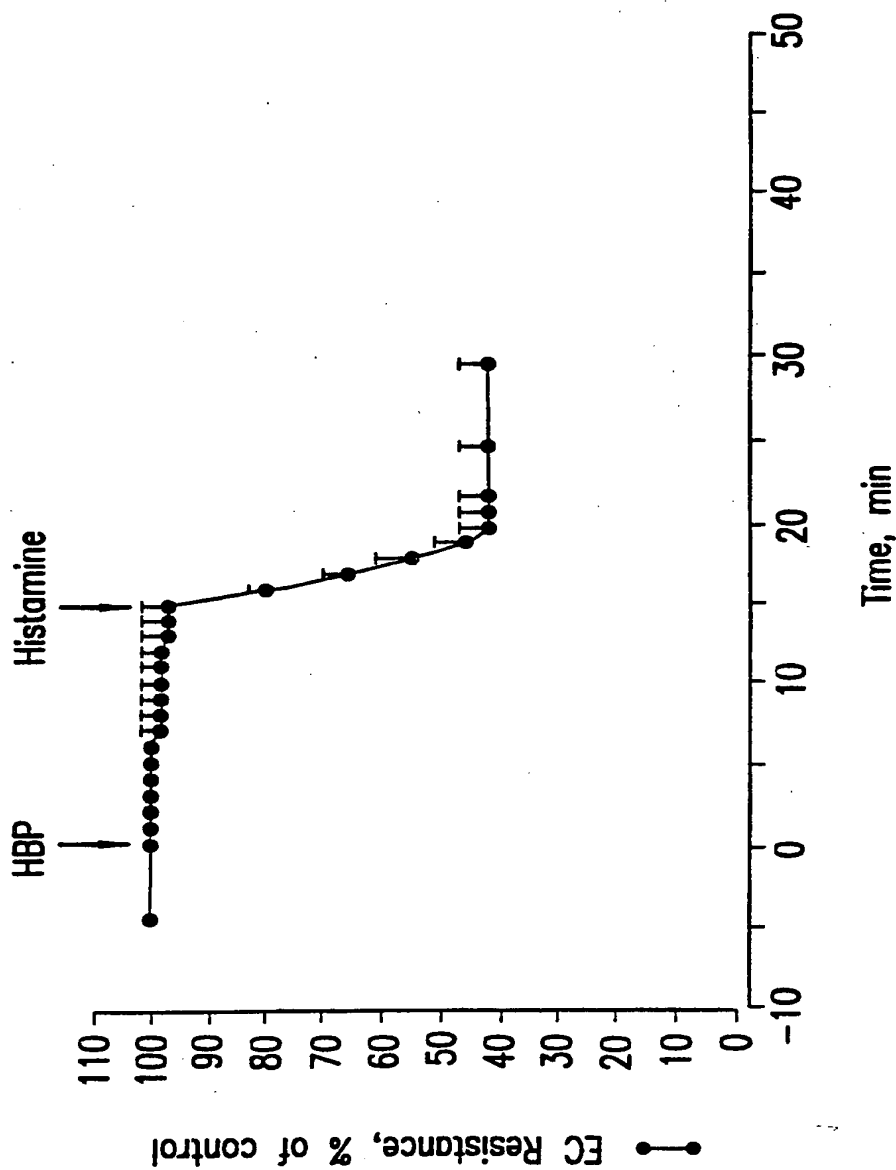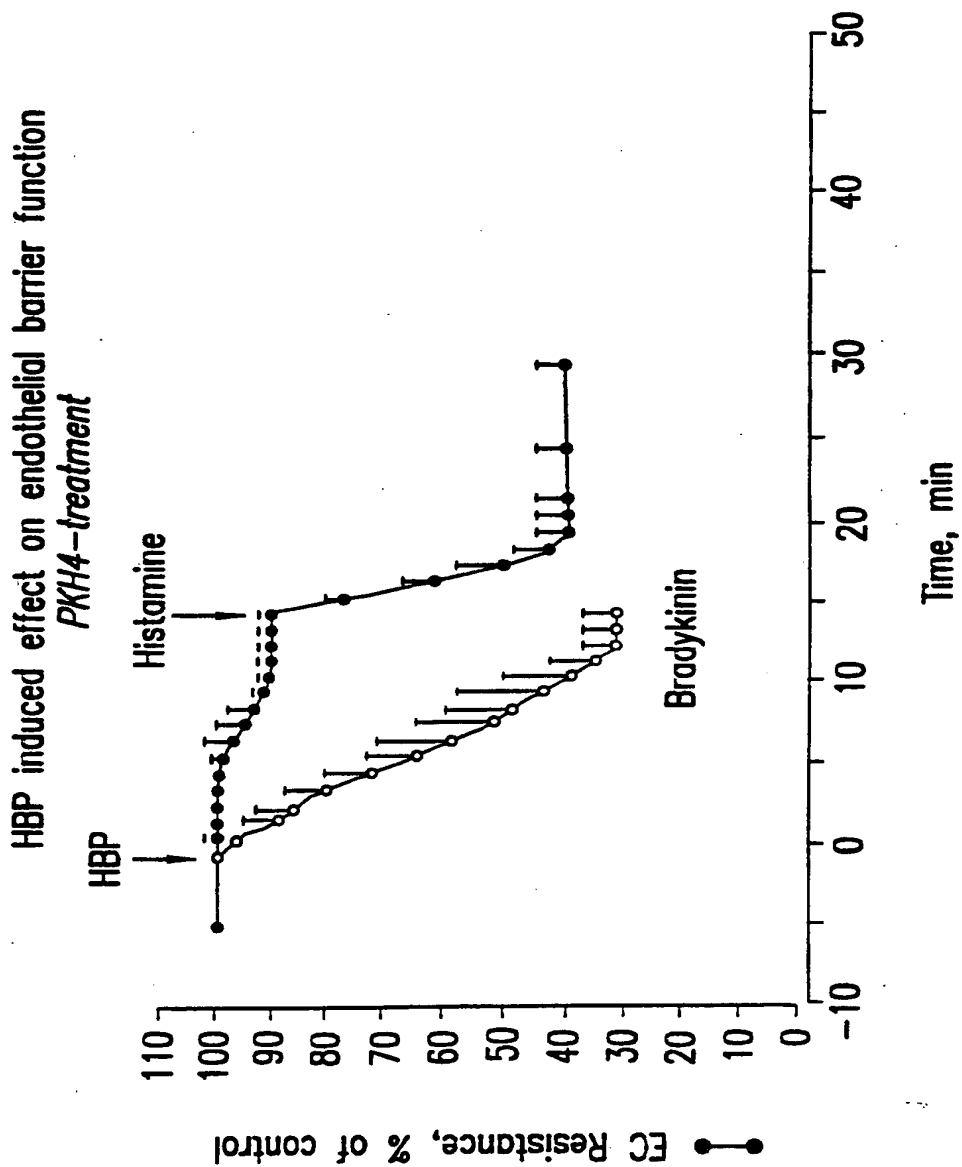
FIG.5b

FIG.6

HBP induced effect on endothelial barrier function
HKH20 treatment
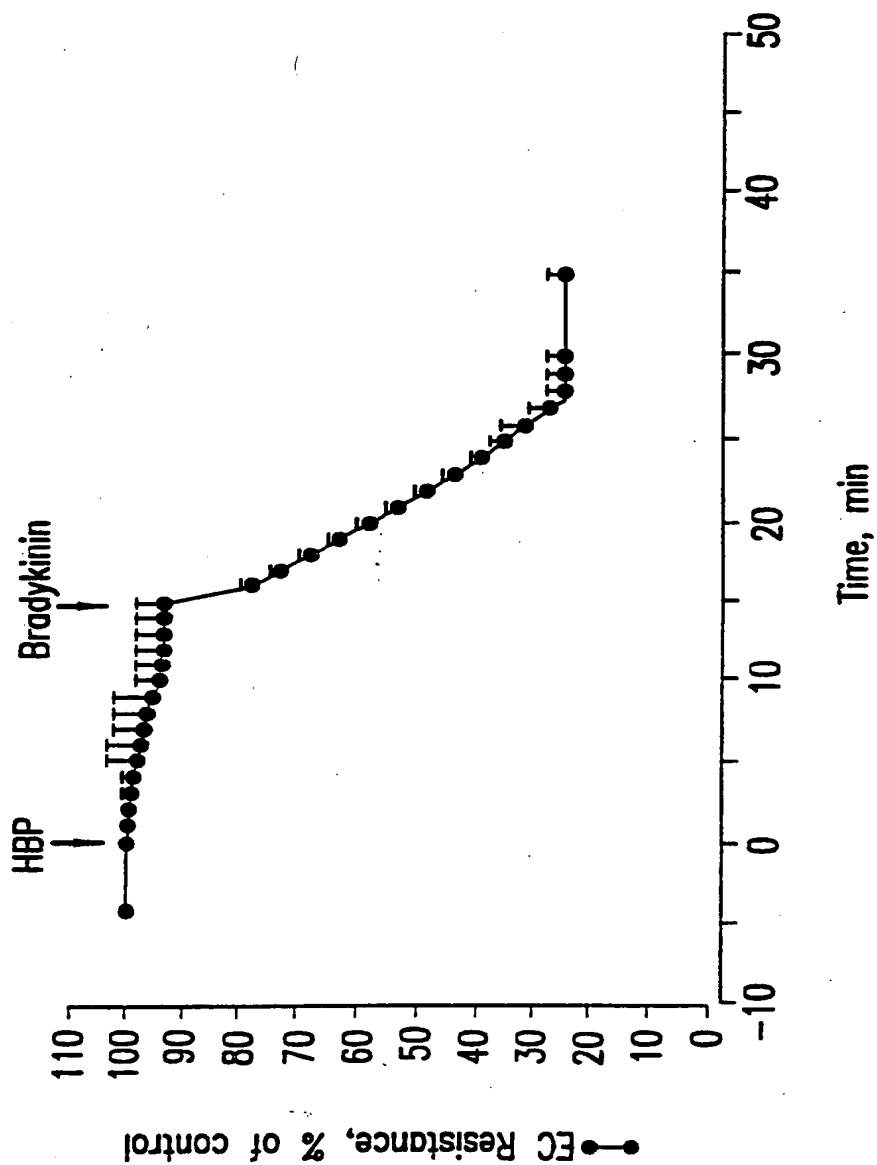
FIG.7

HBP induced effect on endothelial barrier function
HK31/SDD31 treatment
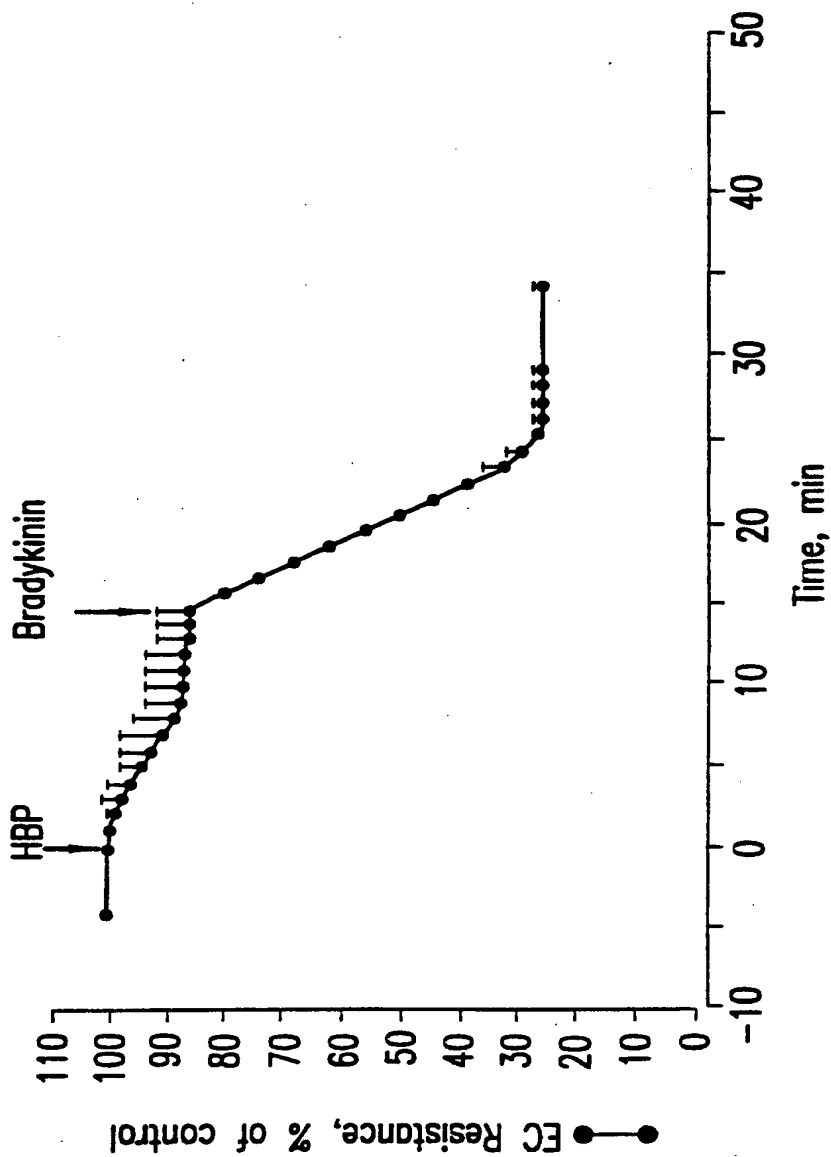
FIG.8

HBP induced changes in EC barrier function
APROTININ treatment
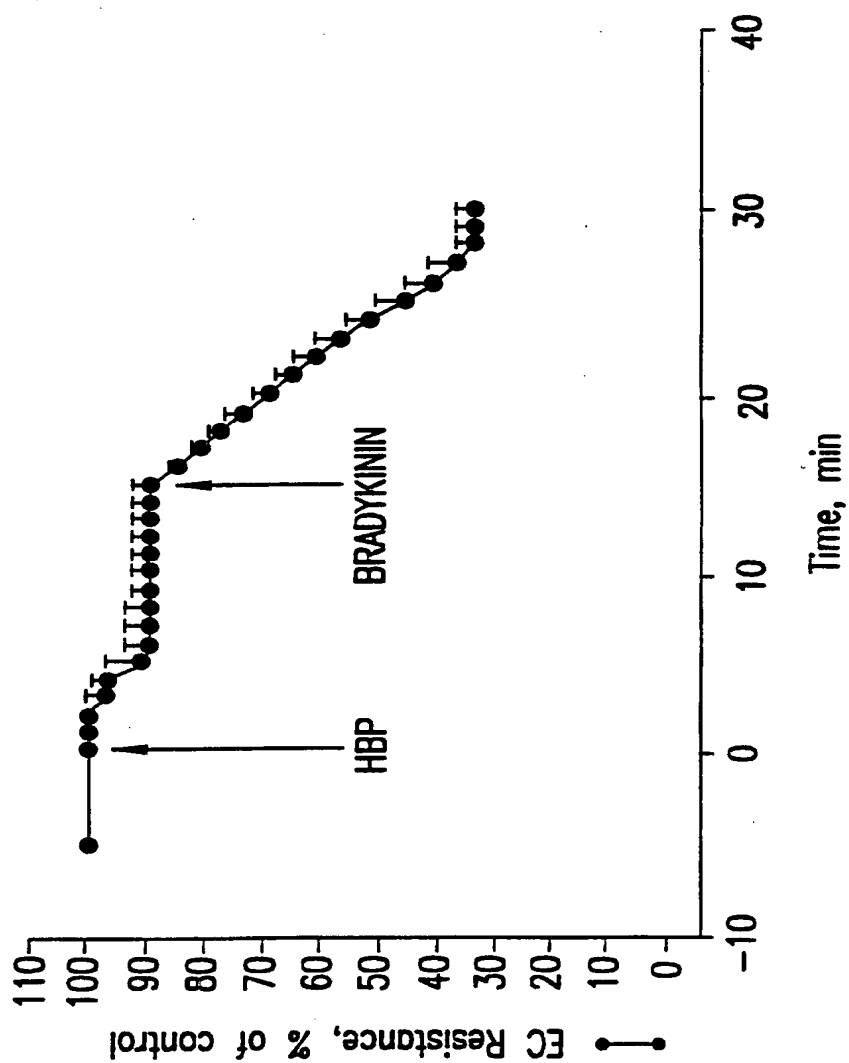
FIG.9
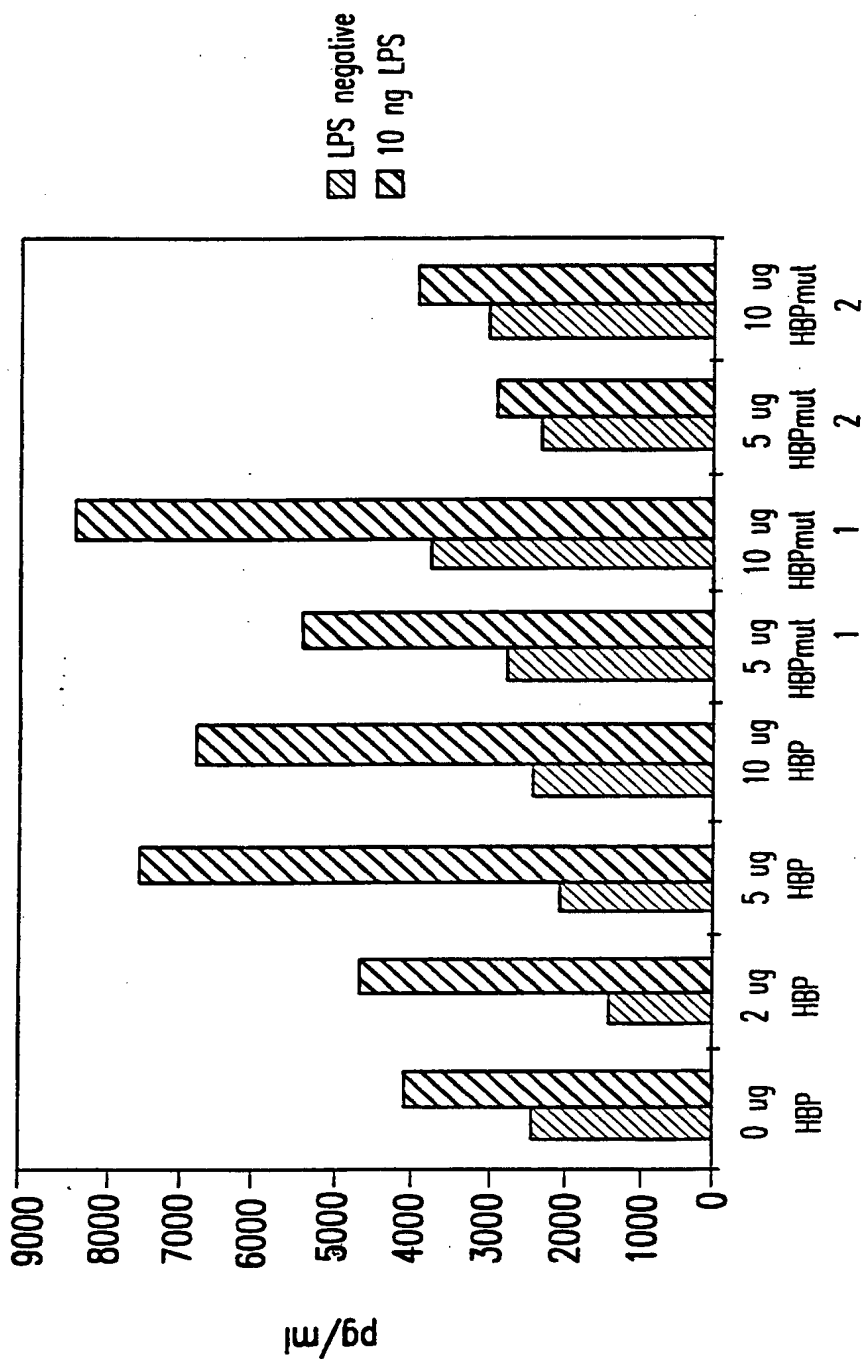
FIG.10

FIG. 11a

FIG. 11b

FIG. 11c

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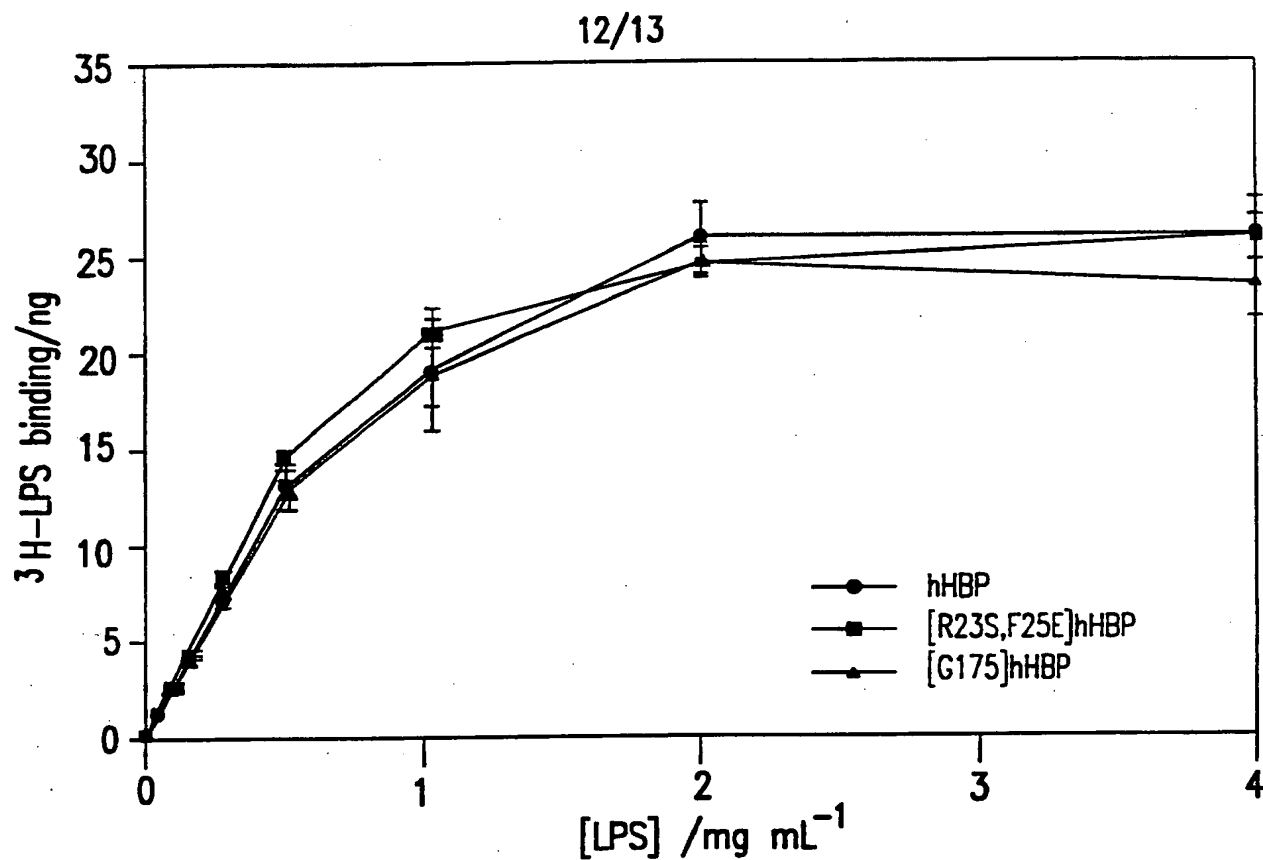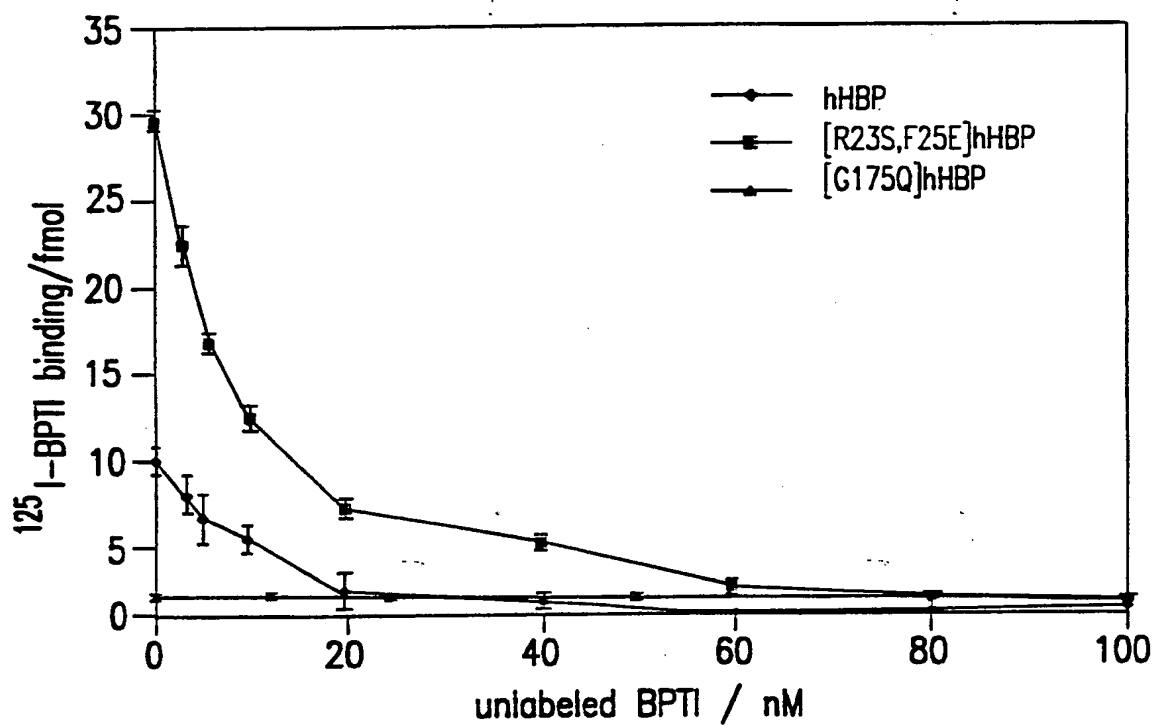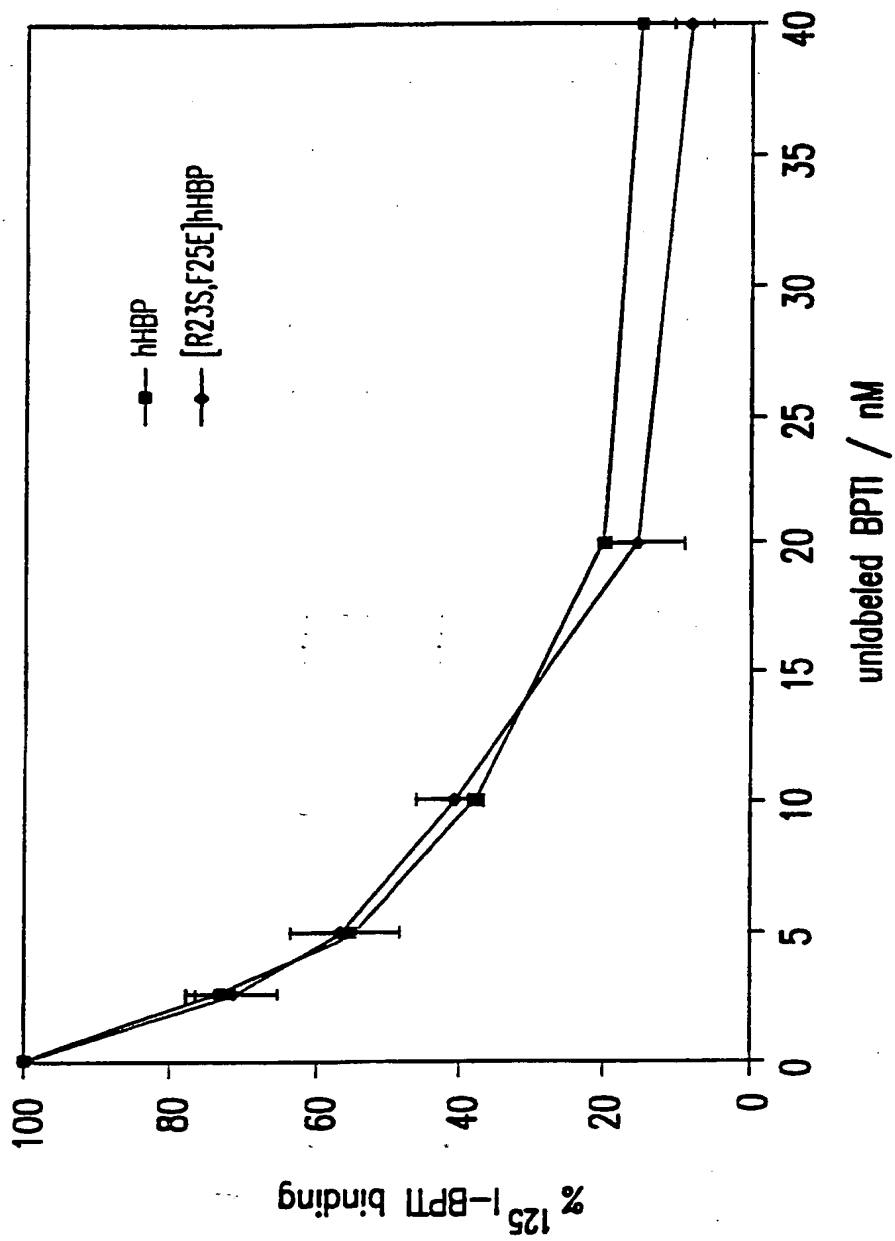
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    50   55   60
Arg Gln Ser Arg Gln Thr Phe Ser Ile Ser Ser Met Ser Glu Asn Gly
65   70   75   80
Tyr Asp Pro Gln Gln Asn Leu Asn Asp Leu Met Leu Leu Gln Leu Asp
                85   90   95
Arg Glu Ala Asn Leu Thr Ser Ser Val Thr Ile Leu Pro Leu Pro Leu
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Gln Asn Ala Thr Val Glu Ala Gly Thr Arg Cys Gln Val Ala Gly Trp
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2

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145                 150             155                 160
Thr Gly Val Leu Thr Arg Arg Gly Gly Ile Cys Asn Gly Asp Gly Gly
            165             170             175
Thr Pro Leu Val Cys Glu Gly Leu Ala His Gly Val Ala Ser Phe Ser
            180             185             190
Leu Gly Pro Cys Gly Arg Gly Pro Asp Phe Phe Thr Arg Val Ala Leu
        195             200             205
Phe Arg Asp Trp Ile Asp Gly Val Leu Asn Asn Pro Gly Pro Gly Pro
    210             215             220
Ala
225
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        <210> 2
        <211> 678
        <212> DNA
        <213> Homo sapiens

        <400> 2
atcgttggcg gccggaaggc gaggccccgc cagttcccgt tcctggcctc cattcagaat      60
caaggcaggc acttctgcgg gggtgccctg atccatgccc gcttcgtgat gaccgcggcc     120
agctgcttcc aaagccagaa ccccgggggtt agcaccgtgg tgctgggtgc ctatgacctg     180
aggcggcggg agaggcagtc ccgccagacg ttttccatca gcagcatgag cgagaatggc     240
tacgacccccc agcagaacct gaacgacctg atgctgcttc agctggaccg tgaggccaac     300
ctcaccagca gcgtgacgat actgccactg cctctgcaga acgccacggt ggaagccggc     360
accagatgcc aggtggccgg ctggggggagc cagcgcagtg ggggggcgtct ctcccgtttt     420
cccaggttcg tcaacgtgac tgtgaccccc gaggaccagt gtcgccccaa caacgtgtgc     480
accggtgtgc tcacccgccg cggtggcatc tgcaatgggg acggggggcac ccccctcgtc     540
tgcgagggcc tggcccacgg cgtggcctcc ttttccctgg ggccctgtgg ccgaggcccct     600
gacttcttca cccgagtggc gctcttccga gactggatcg atggcgtttt aaacaatccg     660
ggaccggggggc cagcctag                                                 678
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        <210> 3
        <211> 698
        <212> DNA
        <213> Homo sapiens

        <400> 3
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tcctggcctc cattcagaat caaggcaggc acttctgcgg gggtgccctg atccatgccc     120
gcttcgtgat gaccgcggcc agctgcttcc aaagccagaa ccccgggggtt agcaccgtgg     180
tgctgggtgc ctatgacctg aggcggcggg agaggcagtc ccgccagacg ttttccatca     240
gcagcatgag cgagaatggc tacgacccccc agcagaacct gaacgacctg atgctgcttc     300
agctggaccg tgaggccaac ctcaccagca gcgtgacgat actgccactg cctctgcaga     360
acgccacggt ggaagccggc accagatgcc aggtggccgg ctgggggagc cagcgcagtg     420
gggggcgtct ctcccgtttt cccaggttcg tcaacgtgac tgtgaccccc gaggaccagt     480
gtcgccccaa caacgtgtgc accggtgtgc tcacccgccg cggtggcatc tgcaatgggg     540
```

3

```
acggggggcac cccccctcgtc tgcgagggcc tggcccacgg cgtggcctcc ttttccctgg    600
ggccctgtgg ccgaggccct gacttcttca cccgagtggc gctcttccga gactggatcg    660
atggcgtttt aaacaatccg ggaccggggc cagcctag                             698
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    <210> 4
    <211> 232
    <212> PRT
    <213> Homo sapiens
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Arg Gln Phe Pro Phe Leu Ala Ser Ile Gln Asn Gln Gly Arg His Phe
            20                  25                  30
Cys Gly Gly Ala Leu Ile His Ala Arg Phe Val Met Thr Ala Ala Ser
        35                  40                  45
Cys Phe Gln Ser Gln Asn Pro Gly Val Ser Thr Val Val Leu Gly Ala
    50                  55                  60
Tyr Asp Leu Arg Arg Arg Glu Arg Gln Ser Arg Gln Thr Phe Ser Ile
65                  70                  75                  80
Ser Ser Met Ser Glu Asn Gly Tyr Asp Pro Gln Gln Asn Leu Asn Asp
                85                  90                  95
Leu Met Leu Leu Gln Leu Asp Arg Glu Ala Asn Leu Thr Ser Ser Val
            100                 105                 110
Thr Ile Leu Pro Leu Pro Leu Gln Asn Ala Thr Val Glu Ala Gly Thr
        115                 120                 125
Arg Cys Gln Val Ala Gly Trp Gly Ser Gln Arg Ser Gly Gly Arg Leu
    130                 135                 140
Ser Arg Phe Pro Arg Phe Val Asn Val Thr Val Thr Pro Glu Asp Gln
145                 150                 155                 160
Cys Arg Pro Asn Asn Val Cys Thr Gly Val Leu Thr Arg Arg Gly Gly
                165                 170                 175
Ile Cys Asn Gly Asp Gly Gly Thr Pro Leu Val Cys Glu Gly Leu Ala
                180                 185                 190
His Gly Val Ala Ser Phe Ser Leu Gly Pro Cys Gly Arg Gly Pro Asp
            195                 200                 205
Phe Phe Thr Arg Val Ala Leu Phe Arg Asp Trp Ile Asp Gly Val Leu
    210                 215                 220
Asn Asn Pro Gly Pro Gly Pro Ala
225                 230
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    <210> 5
    <211> 756
    <212> DNA
    <213> Homo sapiens
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tccagcccccc ttttggacat cgttggcggc cggaaggcga ggccccgcca gttcccgttc    120
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4

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ctggcctcca ttcagaatca aggcaggcac ttctgcgggg gtgccctgat ccatgcccgc      180
ttcgtgatga ccgcggccag ctgcttccaa agccagaacc ccggggttag caccgtggtg      240
ctgggtgcct atgacctgag gcggcgggag aggcagtccc gccagacgtt ttccatcagc      300
agcatgagcg agaatggcta cgaccccag cagaacctga cgacctgat gctgcttcag        360
ctggaccgtg aggccaacct caccagcagc gtgacgatac tgccactgcc tctgcagaac      420
gccacggtgg aagccggcac cagatgccag gtggccggct gggggagcca gcgcagtggg      480
gggcgtctct cccgttttcc caggttcgtc aacgtgactg tgaccccccga ggaccagtgt     540
cgccccaaca cgtgtgcac cggtgtgctc acccgccgcg gtggcatctg caatggggac       600
gggggcaccc ccctcgtctg cgagggcctg cccacggcg tggcctcctt ttccctgggg       660
ccctgtggcc gaggccctga cttcttcacc cgagtggcgc tcttccgaga ctggatcgat      720
ggcgttttaa acaatccggg accggggcca gcctag                                756
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        <210> 6
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        <212> PRT
        <213> Homo sapiens

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Ser Arg Ala Gly Ser Ser Pro Leu Leu Asp Ile Val Gly Gly Arg Lys
            20   25   30
Ala Arg Pro Arg Gln Phe Pro Phe Leu Ala Ser Ile Gln Asn Gln Gly
        35   40   45
Arg His Phe Cys Gly Gly Ala Leu Ile His Ala Arg Phe Val Met Thr
    50   55   60
Ala Ala Ser Cys Phe Gln Ser Gln Asn Pro Gly Val Ser Thr Val Val
65   70   75   80
Leu Gly Ala Tyr Asp Leu Arg Arg Arg Glu Arg Gln Ser Arg Gln Thr
                85   90   95
Phe Ser Ile Ser Ser Met Ser Glu Asn Gly Tyr Asp Pro Gln Gln Asn
                100   105   110
Leu Asn Asp Leu Met Leu Leu Gln Leu Asp Arg Glu Ala Asn Leu Thr
            115   120   125
Ser Ser Val Thr Ile Leu Pro Leu Pro Leu Gln Asn Ala Thr Val Glu
        130   135   140
Ala Gly Thr Arg Cys Gln Val Ala Gly Trp Gly Ser Gln Arg Ser Gly
145   150   155   160
Gly Arg Leu Ser Arg Phe Pro Arg Phe Val Asn Val Thr Val Thr Pro
                165   170   175
Glu Asp Gln Cys Arg Pro Asn Asn Val Cys Thr Gly Val Leu Thr Arg
                180   185   190
Arg Gly Gly Ile Cys Asn Gly Asp Gly Gly Thr Pro Leu Val Cys Glu
            195   200   205
Gly Leu Ala His Gly Val Ala Ser Phe Ser Leu Gly Pro Cys Gly Arg
        210   215   220
Gly Pro Asp Phe Phe Thr Arg Val Ala Leu Phe Arg Asp Trp Ile Asp
225   230   235   240
Gly Val Leu Asn Asn Pro Gly Pro Gly Pro Ala
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5

245                          250

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<210> 7
<211> 19
<212> PRT
<213> Homo sapiens

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His Lys His Gly His Gly His His Lys Lys Asn Lys Gly Lys Lys Asn
1                 5                 10                15
Gly Lys His

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<210> 8
<211> 31
<212> PRT
<213> Homo sapiens

<400> 8
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Ser Asp Asp Asp Trp Ile Pro Asp Ile Gln Thr Asp Pro Asn Gly Leu
1                 5                 10                15
Ser Phe Asn Pro Ile Ser Asp Phe Pro Asp Thr Thr Ser Pro Lys
            20                25                30

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<210> 9
<211> 33
<212> DNA
<213> Artificial Sequence

<220>
<223> pcr primers

<400> 9
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<210> 10
<211> 33
<212> DNA
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<220>
<223> pcr primers

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<212> DNA
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6

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    <220>
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    <211> 27
    <212> DNA
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    <220>
    <223> pcr primers

    <400> 12
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    <210> 13
    <211> 27
    <212> DNA
    <213> Artificial Sequence

    <220>
    <223> pcr primers

    <400> 13
tgcaatgggg accagggcac ccccctc   27

    <210> 14
    <211> 33
    <212> DNA
    <213> Artificial Sequence

    <220>
    <223> pcr primers

    <400> 14
ccggggatcc aactaggctg gccccggtcc cgg   33

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 00/00213

A. CLASSIFICATION OF SUBJECT MATTER

IPC7: A61K 38/55, C07K 16/28, C07K 14/81, G01N 33/53, A61K 39/395, A61P 29/00
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC7: C07K, G01N, A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Biochem J., Volume 311, 1995, Walter A. PATTON II et al, "Identification of a heparin-binding protein using monoclonal antibodies that block heparin binding to porcine aortic endothelial cells" page 461 - page 469	1-30
X		31-58
	--	
A	WO 9305396 A1 (NOVO NORDISK A/S), 18 March 1993 (18.03.93)	1-30
X		31-58
	--	

X	Further documents are listed in the continuation of Box C.		X	See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" erlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
4 Sept 2000	0 8 -09- 2000

Name and mailing address of the ISA/	Authorized officer
Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Carl-Olof Gustafsson/gh Telephone No. +46 8 782 25 00

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.

PCT/DK 00/00213

C (Continuation).   DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Eur J. Biochem, Volume 214, 1993, Lars C. PETERSEN et al, "Binding of bovine pancreatic trypsin inhibitor to heparin bindin g protein/CAP37/azurocidin" page 271 - page 279 --	1-58
X	US 5786328 A (MARK S. DENNIS ET AL), 28 July 1998 (28.07.98), column 1 - column 4; column 22, line 48 - column 23, line 35 --	1-15,17-19, 21,22
X	US 5780265 A (MARK S. DENNIS ET AL), 14 July 1998 (14.07.98), see columns 4 and 2 -- --------	1-15,17-19, 21,22

INTERNATIONAL SEARCH REPORT

Box I	Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 1-7,15-18,23-25
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 1-7,15-18,23-25 are directed to a method of treatment of the human/animal body the search has been carried out based of the alleged effects of the composition (c.f PCT rule 39.1 (iv))

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).:

Box II	Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest   ☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Patent document cited in search report			Publication date	Patent family member(s)			Publication date
WO	9305396	A1	18/03/93	AU	2658092	A	05/04/93
				EP	0645016	A	29/03/95
				JP	7504081	T	11/05/95
US	5786328	A	28/07/98	NONE			
US	5780265	A	14/07/98	AU	6048296	A	24/12/96
				CA	2220130	A	12/12/96
				EP	0832232	A	01/04/98
				IL	122116	D	00/00/00
				JP	11511963	T	19/10/99
				WO	9639519	A	12/12/96